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The lactase enzyme: attempted induction in mammalian cell lines

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INTRODUCTION

The lactose in milk is a principal source of calories for children and this disaccharide is important food for adult nutrition in many areas of the world. However, the ability of some children and adults to digest lactose is severely limited because they totally lack or only have trace amounts of the intestinal enzyme, lactase, a specific intestinal beta-galactosidase.

Milk is a nearly complete human food and in powdered form can be conveniently stored and shipped long distances. Therefore, it is a popular source of protein and other nutrients in many programs of aid to nutritionally impoverished children including American Blacks. The discovery that many of these children are physiologically unable to utilize lactose is thus a matter of grave concern and its implications are being investigated by such agencies as the United States Office of Child Development and the Protein Advisory Group of the United Nations.

In persons possessing intestinal lactase, lactose is hydrolyzed into glucose and galactose. These components are then absorbed by the epithelial cells of the small intestine and transmitted to the blood stream. Individuals that suffer from lactase deficiency are unable to digest lactose and become ill after consuming it. The malady that results from an inability to digest lactose is referred to as lactose intolerance.

The enzyme is not present in the intestine of the embryo or fetus until the middle of the last stages of gestation. Its activity attains a maximum instantly after birth. Thereafter, it begins to decrease reaching

extremely low levels after one and a half to three years in most children. The exact mechanisms involved in the appearance and disappearance of the enzyme activity are not known.

Lactase is also present in mammals other than man in the fetus before birth and in infancy. In these animals the enzyme, as in the human, has its highest activity immediately after birth and begins to decline rapidly reaching very low levels instantly after weaning. The "normal" condition for mammals is not to have the enzyme as adults.

The nucleus of a cell has many functions. One of the most important is the process of gene transcription whereby information encoded in the deoxyribonucleic acid (DNA) is utilized in the synthesis of complementary molecules of ribonucleic acid (RNA). It is through the synthesis of messenger RNA (mRNA) that the genetic information stored in the nuclear DNA is ultimately transmitted to the protein synthesizing machinery of the cytoplasm. The coded message of mRNA is used to direct the translational synthesis of specific proteins.

Specific regulation of the process of gene transcription is extremely important during both the development and normal functioning of cells of higher organisms. For example, differentiated cells that perform different functions utilize characteristic sets of genetic information, and so must use different regions of the genome for the synthesis of mRNA (Stein et al., 1974). Since the DNA of each cell in a multicellular organism appears to be the same, this means that various cell types use the information contained in this DNA differently. Specific regulatory mechanisms must, therefore, be available for activating

particular regions of the genome for mRNA synthesis, depending on the needs of the cell.

In humans the functional lactase activity is "turned on" in the fetus in the latter stages of gestation and abruptly "turned off" in early childhood. For the purposes of this study it was assumed that the gene(s) coding for the lactase enzyme in mammalian cells are under some form of regulatory genetic control.

The objective of this study was to establish a mammalian cell line, in culture, that actively produces the lactase enzyme. Such a cell system would permit extensive investigation of the nature of this mammalian disaccharidase and give some insight into the control systems regulating its cellular synthesis.

All lines of cultured mammalian cells perform a variety of biosynthetic activities, including synthesis of purine and pyrimidine nucleotides, DNA, RNA, seven of the 20 structural amino acids and both structural and enzymatic proteins. Many enzymes can be assayed and their kinetic properties, inhibition characteristics and thermal stabilities studied. The enzymatic defects associated with a number of inherited metabolic defects have been demonstrated in work with cell cultures.

The lactase enzyme is ordinarily present only in the mucosal (epithelial) cells of the small intestine. In an attempt to establish a mammalian cell line capable of producing the lactase enzyme, the following approaches were used:

1. An attempt was made to reverse this natural lactase differentiation (expression of the enzyme only in epithelial cells of the small in-

testine) by using another cell type, an established white blood cell line, in which the genetic potential for lactase production is present although not expressed. These latter cells were exposed to both mutagenic agents and chemical compounds that have been shown to have varying degrees of control over certain differentiated cell characteristics in some mammalian cells grown in culture.

2. An attempt was made to provoke lactase activity in a previously established small intestinal cell line. The cells of this line, maintained in culture since 1957, were derived from cells which naturally would have been expected to produce the lactase enzyme *in vivo*. Enzyme induction was attempted by treatment of the cells with lactose and other enzyme inducers.
3. An attempt was made to establish a new small intestinal epithelial cell line from fetal mice. These cells would be expected to retain their natural *in vivo* lactase activity *in vitro*.

REVIEW OF LITERATURE

The Lactase Enzyme and Lactose Intolerance

Physicians have long recognized that certain individuals do not tolerate milk, that they become ill after consuming it. Though such illness has frequently been diagnosed simply as milk allergy, today the illness can usually be traced to other forms of milk intolerance. One of the causes of milk intolerance is related to the inability of the body to hydrolyze lactose. This form of intolerance is occasionally detected in infants, and some researchers suspect that it is an acquired defect, secondary to acute gastroenteritis; however, with age, such infants are usually able to tolerate larger quantities of milk without distress. Intolerance also occurs in adults, who in infancy and childhood, drank milk without ill effect. This adult type of intolerance, which has its onset after weaning and its origin in lactose malabsorption, is the subject of this review.

The lactase enzyme

In 1963, (Dahlqvist *et al.*; Auricchio *et al.*) it was conclusively proved by intestinal biopsy that adult lactose malabsorption is caused by a deficiency of the enzyme, lactase, (a specific intestinal beta-galactosidase) in the small intestine where lactose digestion and absorption occur. Subsequent studies on biopsy material of human small intestines indicated that there are three intestinal beta-galactosidases (Asp and Dahlqvist, 1972; Asp, 1965; Asp *et al.*, 1969; Gray and Santiago, 1969; Gray *et al.*, 1969).

The first enzyme, the lactase, is a brush border (microvilli) enzyme with an optimum pH 5.5-6.0 and a molecular weight of approximately 280,000. Lactose is the preferential substrate. The second enzyme, the acid beta-galactosidase is thought to be lysosomal and has an optimum pH of 4.0-4.5 and a molecular weight of 156,000-600,000. It has the broadest substrate specificity of the three enzymes hydrolyzing a number of synthetic beta-galactosides (O-nitrophenyl-B-galactoside, 6-bromo-2-naphtyl-B-galactoside, P-nitrophenyl-B-galactoside) as well as lactose *in vitro*. The third enzyme, the hetero-B-galactosidase has optimum pH 5.5-6.0 and a molecular weight of 80,000. This enzyme hydrolyzes synthetic B-galactosides but not lactose. Similar enzymes have also been found in calf (Heilskov, 1956), rabbit (Doell and Kretchmer, 1962) and rat (Dahlqvist *et al.*, 1965; Koldovsky and Chytil, 1965; Koldovsky *et al.*, 1965).

Density gradient ultracentrifugation of intestinal material from lactose intolerant patients (Gray *et al.*, 1969; Asp and Dahlqvist, 1972) demonstrated the absence of a beta-galactosidase with an optimum pH of 5.5-6.0 and a molecular weight of 280,000 which was therefore assumed to be lactase. The other two enzymes were found at normal levels. These studies showed that primary adult lactose intolerance is most probably due to a deficiency of brush border lactase. Since enzyme three has a pH optimum identical with that of the lactase, Gray *et al.* (1969) suggested that this enzyme may be a precursor of lactase. However, there is no evidence to support this suggestion.

The lactase enzyme is not present in the intestine of the mammalian embryo or fetus until the middle of gestation (Kretchmer, 1972). Its

activity attains a maximum immediately after birth. Thereafter it decreases, reaching a low level immediately after weaning in the rat and after one and a half to three years in most children.

The metabolic function of lactase and lactose intolerance

The primary function of brush border lactase is the breakdown of lactose into its component monosaccharides, glucose and galactose, in the jejunum of the small intestines. In normal individuals, the lactose is hydrolyzed into its constituent monosaccharides and they are then absorbed by the epithelial cells and transmitted to the blood stream. Thus, at some time shortly after ingestion of lactose by individuals who possess brush border lactase, there would normally be a measurable rise in the amount of glucose and galactose in the blood. This provides one means of determining whether an individual suffers from lactase deficiency. If a person is suspected of having a lactase deficiency he is administered a lactose tolerance test (LTT). The presence of less than 20 mg/100 ml of blood glucose following an oral dose of 50 grams of lactose usually indicates lactase deficiency. One would expect a considerable rise in blood glucose if lactose were utilized. This is confirmed by a glucose-galactose test. If the patient is fed these two monosaccharides as such, and then experiences a significant rise in blood sugar, it demonstrates that he is able to absorb the hydrolyzed components of lactose, and implies that his difficulty is an inability to hydrolyze lactose because of lactase deficiency. A second means of demonstrating lactase deficiency is biopsy of the intestinal mucosa and direct ascertainment of the amount of

lactase present. A third, less frequently used means, is interpretation of an abdominal radiograph taken an hour after the subject ingests a lactose-barium sulphate mixture. Generally, the radiograph of a lactase deficient person is quite distinctive (Simoons, 1969).

If the amount of lactose presented to the intestinal cells exceeds the hydrolytic capacity of the available lactase, a portion of the lactose remains undigested. Some of it passes into the blood and eventually is excreted in the urine. The remainder moves on into the large intestine where two processes ensue. One is physical. The lactose molecules increase the particle content of the intestinal fluid compared with the fluid in cells outside the intestine and therefore by osmotic action draw water out of the tissues into the intestine. The other process is biochemical. The lactose is fermented by the bacteria in the colon. Organic acids and carbon dioxide are generated and the symptoms are a bloating feeling, flatulence, belching, cramps, and a watery explosive diarrhea (Kretchmer, 1972).

Any disease such as sprue and celiac disease that injures the mucosa of the small intestine also injures the disaccharidases, among them lactase, that are located there. Thus, an incidental adult lactase deficiency may develop when an individual contracts such a disease; and, in tropical sprue it may persist long after other symptoms of the disease have returned to normal (Bayless et al., 1964; Gray et al., 1968). This review is concerned only with the primary form of adult lactase deficiency that occurs in healthy individuals who have every indication of normalcy of the small intestinal mucosa, who have no history of intestinal disease

or damage, and who have other disaccharidases in normal quantities.

The adult who suffers from primary lactose intolerance can consume small quantities of milk, as in tea or coffee, without difficulty. Though individuals differ in the amount of milk needed to bring on symptoms, one or more glasses may cause abdominal distension, discomfort and pains in the malabsorber, and larger quantities may cause diarrhea, with or without cramps. Since an individual would normally relate milk intake to his symptoms, which generally begin 30 to 90 minutes after consumption, he would usually eliminate milk from his diet or confine consumption to manageable amounts which would end his symptoms. Others would avoid fresh milk and instead consume those products such as yogurt, butter and ghee in which the lactose has been broken down by bacterial fermentation or has been removed by washing, thus permitting consumption without ill effect.

The nutritional effects of reduction in milk consumption by the lactose intolerant individual have been investigated but far more study is needed. The lactose intolerant individual is denied the protein and calcium from milk forcing him to supplement from other sources or suffer deficiency. In addition to being an energy source, lactose has been shown to aid calcium absorption (Simoons, 1969). It has been suggested that the disease, osteoporosis, is associated with reduction of milk consumption by the intolerant individual.

Group differences in lactose tolerance

The earliest research on lactase deficiency carried out mainly in the United States and in Europe led to the erroneus belief that in normal humans everywhere lactase production remains at a high level through

life which permits milk consumption without adverse effect regardless of age. This view is found in the literature at least into the mid 1960's (Haemmerli et al., 1965; Haemmerli and Kistler, 1966, 1967). Since this time researchers have become aware of significant group differences among adults in lactase deficiency and in lactose intolerance.

In the United States, where much of the pioneering research of the late 1960's was done at the John Hopkins University School of Medicine, striking differences were demonstrated between white and Negro adults. One study (Bayless and Rosensweig, 1966, 1967; Rosensweig and Bayless, 1966) of 40 healthy, volunteer prisoners, 20 white and 20 Negro, who were chosen consecutively, showed that 95% of the Negroes but only 10 percent of the whites reported having experienced symptoms of milk intolerance in the past; lactose tolerance tests indicated that 90 percent of the Negroes and 10 percent of the whites were lactose malabsorbers; and intestinal biopsies revealed that 70 percent of the Negroes and five percent of the whites were lactase deficient. Most of the milk intolerant individuals reported consuming milk in their youth, many drinking a quart or more daily without ill effect, and they also spoke of an onset of symptoms in adolescence or in their early twenties. A subsequent study (Huang and Bayless, 1967) was made of 20 Black and 20 white children, who ranged in age from 11 months to 11 years, all healthy and chosen at random from children attending a Baltimore pediatric clinic; their families were all of a comparable socioeconomic level. The results were similar. Moreover, data from earlier studies indicated that with age there was a decrease in lactase among healthy Black Americans, whereas most healthy white Americans

had a constant level of lactase regardless of age.

Another study (Welsh et al., 1967) of 170 patients from medical centers in Oklahoma, Texas, and Nebraska showed a lactase deficiency, not only significantly higher in Blacks (77 percent) than in whites (19 percent), but also higher in American Indians (67 percent) than in whites. An investigation of 20 healthy adult Orientals (Huang and Bayless, 1968) residing in the United States revealed that 70 percent of the subjects experienced symptoms of malabsorption after ingesting one or two glasses of milk; and that 95 percent had symptoms after consuming lactose equal to the amount contained in a quart of milk. All the Orientals had drunk milk without symptoms as infants and most reported the onset of symptoms in adolescence or later. Of a control group of healthy white Americans, only 10 percent were intolerant of lactose. A high incidence of lactase intolerance among healthy Orientals in American was confirmed by lactose tolerance tests. One hundred percent of those tested were found to be lactase deficient (Chung and McGill, 1968).

In a study conducted among various tribal groups in East Africa (Cook and Kajubi, 1966) parallel differences were demonstrated. Lactose tolerance tests were performed on 135 adults and children and lactase determinations were made from intestinal biopsy for 54 of the subjects. Striking differences were found between the primarily agricultural Ganda of Uganda, along with neighboring agricultural tribes who are also of Bantu speech, and certain nearby peoples of nomadic tradition, i.e., the Hamitic Hima of Uganda and Tussi of Rwanda. Analysis of the data presented for Bantu agriculturists and the nomadic Hamites revealed that

the Ganda and their neighbors have a high incidence (90 percent) of adult lactose intolerance, whereas the Hima and Tussi have quite low incidences (9 percent and 16 percent). The agricultural Hutu of Rwanda and the Iru of the Ankule district of Uganda, who are presumed to be of mixed Bantu/Hamitic origin, have intolerance levels (33 to 38 percent) that are intermediate between those of the other Uganda Bantu agricultural tribes and those of the Hamitic peoples. These results were confirmed by intestinal biopsies and subsequent studies of members of the same tribe (Cook, 1967; Cook et al., 1967; Cook and Howels, 1968; Cook and Dahlqvist, 1968).

There have been only a few investigations of lactose intolerance among Asians. In one of these (Davis and Bolin, 1967; Bolin et al., 1968; Bolin and Davis, 1969) however, the results parallel those previously mentioned for Orientals in the United States (Huang and Bayless, 1968; Chung and McGill, 1968). This study involved students living in Australia, among them 30 Chinese, five Indians. Twenty-three asymptomatic white Australians were used as a control group. Whereas no member of the control group proved to be lactose intolerant, 90 percent of the Chinese and 80 percent of the Indians did. Another Indian study by Desai et al. (1967) gave similar results.

Four studies (Halsted et al., 1969; Gilat et al., 1971; Pena Yanez et al., 1971; Rotthauwe et al., 1971) have confirmed the existence of a generally high incidence of lactose intolerance and lactase deficiency among Arabs. These studies indicate that approximately 80 percent of the Arab population suffer from primary adult lactase deficiency.

A high incidence of adult lactose intolerance has also been confirmed among Jews in Israel. Rozen and Shafrir (1968) found that 57 of 93 (61 percent) adult Israeli Jews, mainly clinic patients, had low rises in glucose during the LTT. Gilat et al. (1970) used lactose tolerance tests to check 217 Israeli Jews. Sixty-three of the 217 were also biopsied to determine lactase deficiency. Overall, 71 percent of those given LTT were lactose intolerant, and 60 percent of those biopsied were lactase deficient. Leichter (1971) also reported similar findings among Canadian and American Jews.

In Europe, the only evidence of widespread lactose intolerance among an ethnic group is found among Greeks. Many of them are said to have milk intolerance and in one study, 15 of 17 Greek Cypriots, who were without gastroenterological problems, proved to be lactose intolerant (McMichael et al., 1965). By contrast, of 90 Northwest Europeans (British, Irish, Scandinavian, and German), only 11 persons, or 12 percent were malabsorbers. The latter figure is comparable to that reported for white Americans who are predominantly of Northwest European ancestry.

The above studies (summarized in Table 1) indicate that many more groups all over the world are intolerant to lactose than are tolerant. Real adult tolerance to lactose has so far been observed only in Northern Europeans, approximately 90 percent of whom tolerate lactose, and in the members of two nomadic, pastoral tribes in Africa, of whom about 80 percent are tolerant (Kretchmer, 1972). Although other generally tolerant groups may be found, they can only belong to a minority segment of the human species since Orientals and people of African descent constitute

Table 1. Prevalence of adult lactase deficiency in human population subgroups

Group	Percent Lactase Deficient	Reference
American White	10.0	Bayless and Rosensweig, 1966, 1967; Huang and Bayless, 1967.
American Black	70.0	Bayless and Rosensweig, 1966, 1967; Rosensweig and Bayless, 1966; Welsh et al., 1967.
American Indian	67.0	Welsh et al., 1967.
American Jew	79.0	Leichter, 1971.
Israeli Jew	71.0	Rozen and Shafrir, 1968; Gilat et al., 1970.
Oriental	100.0	Huang and Bayless, 1968; Davis and Bolin, 1967; Bolin et al., 1968; Chung and McGill, 1968.
Indian	80.0	Desai, et al., 1967.
African Bantu	90.0	Cook and Kajubi, 1966; Cook, 1967; Cook et al., 1967.
Arab	80.0	Halstead et al., 1969; Gilat et al., 1971; Pena Yanez et al., 1971; Rotthauwe et al., 1971.
Greek	88.0	McMichael et al., 1965.

the majority of the world's population.

The basis for group differences in lactose tolerance

Among the possible hypotheses for explaining these differences in adult lactose tolerance are the following:

1. That some ethnic groups have a high incidence of the specific diseases which damage the intestinal mucosa and inhibit lactase production, and that, even after affected individuals have recovered and are in apparent good health, the effects remain. If this explanation is correct, group differences in lactose tolerance would reflect differences in the incidence of such diseases (the disease hypothesis).
2. That drugs or other items of diet inhibit lactase production. In this view, group differences in lactose tolerance derive from contrasts in the consumption of such inhibitors (the hypothesis of dietary inhibition).
3. That an individual's production of lactase and his ability to hydrolyze lactose normally decreases with age unless milk is consumed (the hypothesis of lactase induction by milk consumption).
4. That the group differences are genetic in origin (the genetic hypothesis).

The disease hypothesis It is difficult to assess the role of disease in contributing to group differences in lactose tolerance. It is recognized that there is widespread subclinical disease of the small

intestine among symptom-free and seemingly normal individuals in various tropical countries, and that the intestinal mucosa of affected individuals are altered in form and absorptive ability (Lindenbaum et al., 1966; England, 1968; Sheehy et al., 1968). There must be among the world's peoples differences in the incidence of subclinical disease, as well as in the more severe clinical forms of disease that induce malabsorption of lactose (Simoons, 1969). It is also claimed that of all the dissacharidases, lactase is the most readily affected by damage to the intestinal mucosa and is the slowest to recover (Chandra et al., 1968; Holzel, 1968).

One of the diseases in which there commonly is an intolerance of lactose is kwashiorkor, one form of severe protein-calorie malnutrition. Children ill with kwashiorkor almost always have diarrhea, and in Africa it has been demonstrated that in many patients, the diarrhea can be controlled by the removal of milk from the diet (Bowie et al., 1963). The milk intolerance of the African kwashiorkor patient has also been shown to derive from an intolerance of lactose and a deficiency of lactase. It has been suspected (Bowie et al., 1965; Bowie et al., 1967) that the lactase deficiency is secondary to the disease, and that it develops because of intestinal mucosal damage brought on by protein deprivation. Since the lactase deficiency continues to be found in some Africans long after they have recovered from the illness (Bowie et al., 1967; Cook and Lee, 1966), kwashiorkor would seem to contribute to adult lactose intolerance.

However, subsequent studies challenged this and certain other aspects of the kwashiorkor/lactase relationship. Work with rats (Solimano

et al., 1967; Prosper et al., 1968) showed that protein deprivation does not significantly decrease lactase or other intestinal disaccharidase activities. A species difference could be present, or the case may be that something other than protein deprivation is responsible for the lactase deficiency that occurs in kwashiokor. In another study of Indian infants and children suffering from protein-calorie malnutrition, (Chandra et al., 1968) it was found that all but four of the 39 who had been lactose intolerant were able to hydrolyze lactose after recovery from malnutrition. Of the four, three had a parent who was lactose intolerant which suggested that the intolerance was inherited; and there was a possibility that the fourth also had an inherited inability to absorb monosaccharides. The results of this study open the possibility that the African children who had long recovered from kwashiorkor but remained lactose intolerant were so before becoming ill.

Further study is needed before the relationship of kwashiorkor to lactose intolerance is fully understood. It is clear, however, that in whatever way kwashiorkor or other diseases may contribute to group differences in lactose tolerance among adults, this can be only one element in the situation, for another important question remains unanswered. Why should there be significant differences in lactase production and lactose tolerance among groups living in the same environment, both tropical and subtropical, and who have been exposed to similar risks of disease and intestinal damage? For example, Blacks, Orientals and whites living in America or Hamitic and Bantu peoples in East Africa? Why should there be this disparity among these healthy adults who show no

evidence of jejunal abnormality and who can absorb sugars other than lactose without difficulty?

The hypothesis of dietary inhibition This hypothesis, as advanced by Sunshine (1964), is based on the observation that taking the drug, colchicine, decreases an individual's lactase production and that he then becomes ill if he drinks milk. The idea is that foods or drugs may contain similar lactase-inhibiting substances and that the differing ethnic consumption patterns create group differences in lactose tolerance. There is no evidence in the literature to support this theory. A similar concept was set forth by Sprinz et al. (1962). This investigator suggested that highly spiced foods and betel nut consumption may alter the intestinal mucosa and contribute to malabsorption. Since research has not been carried out on diet as a possible contributory factor to group differences in absorption of lactose or other sugars, such hypotheses can only be regarded as being highly speculative.

Hypothesis of lactase induction by milk consumption The questions are: (1) whether it is normal for an individual's lactase production and lactose absorption to decrease after weaning and, if so, whether lactase production can be made to persist throughout life if the individual continues to consume lactose after this time; (2) whether group differences in adult lactose tolerance have their origin in the contrasts of group difference in milk consumption that are evident after weaning.

The possibility of individual adaption to lactose has been considered since the beginning of the century, usually through attempts to relate

lactase activity to the concentration of milk in the diet of nursing animals. A number of animal studies may be found in the literature (Simoons, 1969; Broitmann et al., 1968; Cain et al., 1968; Reddy et al., 1968; Huber et al., 1961; Doell and Kretchmer, 1962; Huber et al., 1964; Fischer and Sutton, 1953; Plimmer, 1906). Unfortunately, the studies have been quite different in design and execution, in the species of animal used, in the amount of lactose fed, in the length of time for which the experiment continued, in the timing of biopsies, in the part of the intestine biopsied, in the method of enzyme assay, and in other ways. All of these differences make it difficult to compare the results.

Despite this difficulty, certain conclusions can be drawn from these experiments: (1) Lactase production continues in the animal intestine even if all lactose is eliminated from the diet (Huber et al., 1961) (2) If an animal continues to receive lactose during and after weaning, there may be a delay in the normal drop of lactase activity with age (Simoons, 1969). (3) Lactase levels in adult animals are much lower than those of their infancy, and it has been demonstrated that throughout life they can not be maintained at a consistently high level by lactose feeding (Simoons, 1969; Doell and Kretchmer, 1962). (4) Lactose feeding can lead to a proliferation of the intestinal mucosa of nursing animals and an increase in the total lactase activity of the small intestine (Huber et al., 1964). (5) Some previously weaned animals have an increase in specific lactase levels as a result of feeding on rations that are extremely high in lactose (Cain et al., 1968; Broitmann et al., 1968). However, (6) it has not been clearly ascertained whether lactose feeding

actually induces activity of the lactase enzyme or whether there is simply a decrease in breakdown as suggested by Reddy et al. (1968).

Simoons (1969) questions the relevance of the animal experiments to the human situation. The lactose of cow's milk, the form of milk most commonly used by man, is usually between 4.5 and 5.0 percent by weight. The animal experiments, however, commonly have involved diets containing 25 or 30 percent lactose, at least five times the amount of lactose in a diet composed only of milk. It is inconceivable that under day to day conditions, man could consume lactose in these amounts.

Some of the evidence on lactase induction in humans is experimental, some circumstantial. Investigations of lactase activity in human fetuses and the newborn (Heilskov, 1951; Auricchio et al., 1965) demonstrate that lactase is not present in the intrauterine stage and increases to high levels shortly before birth. Since the unborn child does not consume milk, this increase in lactase level must be brought about by something other than lactose. With reference to induction of lactase in adults, a number of studies have been done (Cuatrecasas et al., 1965; Knudsen et al., 1968; Kogut et al., 1967; Rosensweig and Herman, 1969; Dunphy et al., 1965; Bayless and Rosensweig, 1966, 1967; Jersky and Kinsley, 1967; Chung and McGill, 1968). These studies suggest the following:

1. That in humans the intestinal lactase level is independent of the amount of lactose consumed.
2. That an absence of milk from the diet does not depress lactase levels, nor does its presence increase it.

3. That among most European peoples the persistence of high levels of lactase into adulthood derives from other causes.

The genetic hypothesis The premise that group differences in adult lactose intolerance are genetic in origin is supported by an overwhelming majority of researchers in medicine and related fields. It is difficult to conceive of an explanation other than genetic that can account so readily for the persistence of high levels of intolerance among Blacks and Orientals in the United States, an environment strikingly different from those of their ancestral homelands.

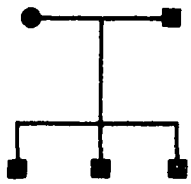
To prove a genetic etiology, complete family histories are needed. At present the literature contains only a few such studies (Ferguson and Maxwell, 1967; Welsh et al., 1968; Flatz and Saengudom, 1969; Welsh, 1970; Gilat et al., 1973). Taken together these studies report a total of only 42 families in which both parents and one or more of their offsprings were subjected to lactose tolerance tests and lactase assay. Representative pedigrees for some of the families studied are given in Figures 1-3. The results of these studies strongly indicate a genetic etiology for primary adult lactose intolerance.

The mode of inheritance is not completely clear. However, it has been suggested by Ferguson and Maxwell (1967) and others (Fine et al., 1968; Flatz and Saengudom, 1969) that primary adult lactase deficiency is inherited as an autosomal recessive trait. Three alleles were postulated: L (normal, dominant), l_1 and l_2 (both recessive). It has been assumed that genotypes LL, Ll_1 and Ll_2 result in the normal phenotype; l_1l_1 in primary adult lactase deficiency and l_2l_2 in the rare alactasia (congen-

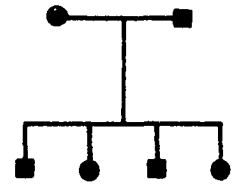
Figure 1. Results of lactose tolerance tests and lactase assays in five pedigrees from Welsh et al. (1968) (A) and Ferguson and Maxwell (1967) (B)

A

Family 1



Family 2



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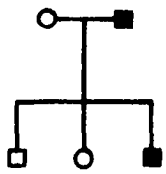

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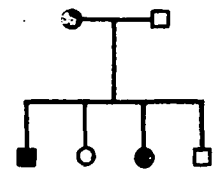
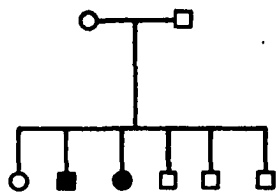


B



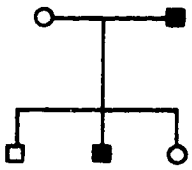
○ Female

□ Male

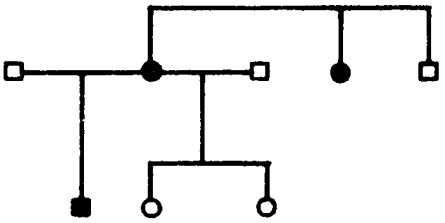
● ■ Lactose Intolerant

Figure 2. Results of lactose tolerance tests and lactase assays in five pedigrees (Welsh, 1970)

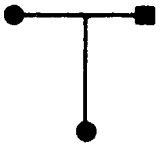
Family A



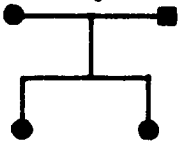
Family B



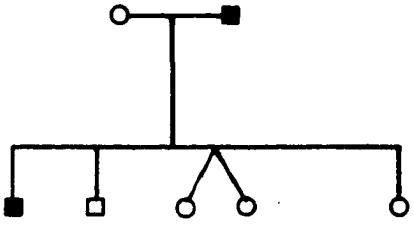
Family C



Family D



Family E



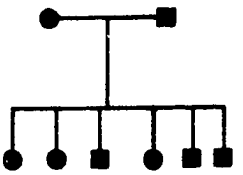
○ Female

□ Male

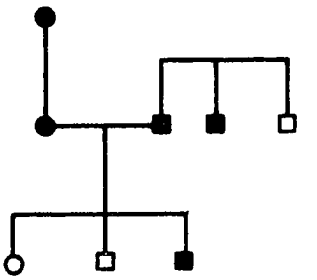
● ■ Lactose Intolerant

Figure 3. Results of lactose tolerance tests and lactase assays in six pedigrees (Gilat et al., 1973)

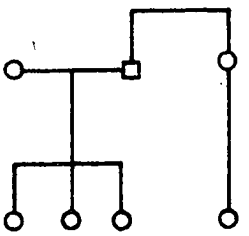
Family 1



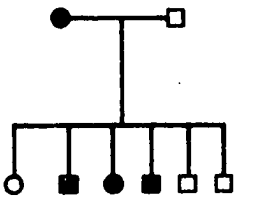
Family 2



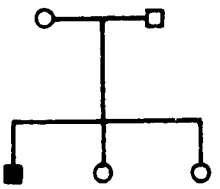
Family 6



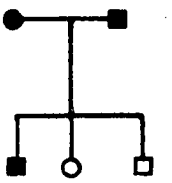
Family 13



Family 17



Family 31



○ Female

□ Male

● ■ Lactose Intolerant

ital lactase deficiency). A trait is usually considered to be inherited as a recessive if it is found in the offspring of parents who lack the trait. There were only two families (Figure 1B and family 17, Figure 3) in the above studies in which this was the case. Data from these two families alone is not sufficient to conclude that primary adult lactase deficiency is inherited as a Mendelian recessive.

The critical families for testing this mode of inheritance would be those in which both parents have lactase deficiency. Being presumably homozygous for the recessive gene, all their offspring should also have lactase deficiency. Welsh et al. (1968) studied two such families (Figure 1, families 1 and 2) and determined that all the siblings were deficient. Welsh (1970) also studied two such families (Figure 2, families C and D) and found all the children to be deficient. However, Gilat et al. (1973) studied 11 such families; in two of the families (Figure 3, families 2 and 31) he found that not all the children were deficient. These findings provide some argument against recessive inheritance, but Gilat and his colleagues suggested that this discrepancy could have been due to an error in interpretation of the lactose tolerance tests or that one of the parents could have had secondary lactase deficiency (deficiency due to intestinal injury). Even if Gilat's arguments should prove true, many more family studies are needed before final statements of recessive inheritance can be made.

The conclusion that can be drawn at present is that the racial differences which have been shown to be correlated with lactose intolerance are almost certainly genetic in origin.

Alterations of Genotypes in Cell Culture

For more than 30 years, it has been known that human and other mammalian cells could be grown *in vitro* much in the same manner as microorganisms. The techniques of tissue and cell culture have now advanced to the point where it is relatively easy for the investigator to study and obtain valuable information relating to a number of diverse biological functions. Among these are growth, differentiation, metabolism, the genetics of mammalian cells, and the metabolic basis of inherited diseases.

One tool applied in studies in the areas mentioned above is the alteration of genotypes of cells grown in culture and the observation of the effect that these alterations or changes have on the particular function in question.

Mutagenesis

Changes in the genotype of cultured mammalian cells are usually induced by treating the cells with chemical mutagens. Chemical mutagens are agents that alter DNA giving rise to mutations (permanent inheritable changes in the genetic material of organisms).

The successful isolation of mutant or variant cells in cell culture requires the following:

1. A cell line that has a relatively high cloning efficiency. Cloning efficiency is the proportion of single cells, inoculated in low density that are capable of multiplying in number to form macroscopically visible colonies. The colonies can then be isolated as clones which are the pure line progeny of single cells.

A cell line with a poor cloning efficiency hinders a reliable estimate of mutation frequency as well as a successful isolation of clones.

2. A chemically defined growth medium. A medium that permits optimum cell growth.
3. A chemical mutagen whose mode of action is known.
4. A selection system. A method of selecting cells of altered genotype following the exposure of a parent population to the mutagenic agent. There are at least four different methods for mutation selection in mammalian cell cultures, all of which are modified from the analogous selective procedures successfully developed in microbial genetics (Chu, 1971). These methods are (a) the mass selection method; (b) the lethal growth method; (c) the thymineless death method; and (d) the replica plating method. The mass selection method was used in this study. This procedure involves simply exposing mass cultures which have been treated with mutagen to extrinsic agents or specifically altered growth media which will not permit growth of nonmutagenized parental cells. The parental cells are nonviable in the selective environment, therefore, the surviving cell population should include variants having altered genotypes and a different phenotype. Variants thus isolated include those having altered carbohydrate metabolism (Hsu and Kellog, 1959).

A variant isolated in mammalian cell culture may not necessarily represent a true genetic alteration. For example, Terzi (1974) found that some apparent drug resistant mutants of mammalian cell lines were not true mutants, but were variants that had survived the selection treatment due to a temporary imbalance in chromosome number. Therefore, the important task after isolation from a selection experiment, is to determine the genetic or epigenetic nature of such variants. For somatic cells the following criteria (Thompson and Baker, 1973) are applied to define mutant:

1. The altered phenotype breeds true in the sense that it is stably transmitted through consecutive generations.
2. The frequency of occurrence of the mutant phenotype can be enhanced by the application of mutagenic agents.
3. The phenotype can ordinarily be associated with an altered gene product (usually a protein).
4. The phenotype can be attributed to a specific region of the genome, i.e., it can be mapped in a chromosomal linkage group.

Chemical compounds that effect differentiation

If DNA directs all cell metabolism, then there are probably two broad categories of DNA activity during differentiation (Bischoff and Holtzer, 1970). First, the DNA codes for those essential molecules common to all cells; the respiratory enzymes and associated structural proteins, the enzymes synthesizing amino acids, common sugars, nucleotides, and all the species of molecules required for cell multiplication.

The second category involves the activation of different sets of nucleotide sequences in subpopulations of cells. It is this mechanism, the turning on or off of certain genes regulating tissue specific "luxury" molecules that is involved in differentiation (Holtzer et al., 1968). Bromodeoxyuridine and dimethyl sulphoxide are chemical compounds that have been shown to have differential effects on these two classes of DNA function. These chemicals were used in this study.

Bromodeoxyuridine (BUDR) BUDR, a pyrimidine analogue, is incorporated into the DNA of dividing cells in place of thymine (Eidinoff et al., 1959; Djordjevic and Szybalski, 1960). The consequence of such a replacement in differentiating cells is a rapid inhibition of the synthesis of tissue specific components. In the presence of BUDR, (1) chick embryo myoblasts fail to fuse and to produce myosin (Stockdale et al., 1964; Bischoff and Holtzer, 1970); (2) chick embryo chondrocytes fail to initiate the production of, or lose the ability to synthesize chrondroitin sulfate and fail to deposit matrix (Abbot and Holtzer, 1968; Coleman et al., 1970); (3) mouse pancreatic cells no longer produce zymogen (Wessells, 1964); (4) chick amnion cells fail to synthesize hyaluronic acid (Bischoff and Holtzer, 1968); (5) rat hepatoma cells fail to synthesize tyrosine aminotransferase when induced by sterols (Stellwagen and Tomkins, 1971a 1971b); (6) chick eye pigment cells and mouse melanoma cells are inhibited in producing melanin (Silagi and Bruce, 1970); and (7) red blood cells fail to synthesize hemoglobin (Miura and Wilt, 1971). These effects are reversible if the exposed cells are grown in BUDR-free media or media supplemented with deoxythymidine.

A typical change in morphology accompanied the above phenomena in all but the rat hepatoma cells and the pancreatic cells. The cells flattened inordinately and adhered more strongly to the culture dish surface, taking on a fibroblastic appearance.

A most conspicuous observation is the apparent lack of effect of low level BUDR on essential cell functions. In general, cell growth and division are not appreciably affected. It appears that those cell functions associated with tissue specific molecules characteristic of a differentiated state are more sensitive to BUDR than are the activities required for growth and cell division.

Several sorts of models have been proposed to explain these differential effects of BUDR. Most proposals postulate that the phenotypic effects of BUDR are a consequence of its replacement for thymine in DNA, and extensive DNA density shifts have been found in myoblasts (Stockdale et al., 1964) and rat hepatoma cells (Stellwagen and Tomkins, 1971a) after BUDR treatment. Really rigorous evidence that incorporation of BUDR into DNA is necessary for its effect is lacking. Evidence from synchronized cells (Wilt and Anderson, 1972) suggest that in some systems events associated with the S period are sensitive to the action of BUDR.

Assuming that incorporation of BUDR into DNA is necessary for its action, models dealing with the phenomena must explain why it is reversible and why there is selective inhibition of certain cellular functions. One hypothesis is that DNA containing BUDR acts as a counterfeit template for synthesis of informational RNA molecules and that improper messages are synthesized (Lasher and Cahn, 1969). This kind of mutagenic action

of BUDR is unlikely. The effects are all-or-none, reversible, not dependent on visible light; and, as far as has been tested, abnormal proteins are not synthesized.

A second proposal postulates that DNA containing BUDR, while transcribed with fidelity, is not transcribed as often as usual. Stellwagen and Tomkins (1971b) suggest that BUDR in DNA inhibits the transcription of only certain genes into messenger RNA. If this postulate is coupled with differential degradation of certain messengers and proteins, the effects of BUDR may be explained. The model is not completely satisfactory, however, because it does not explain why transcription of certain genes is more sensitive than others.

Finally, there is a class of models which postulate that DNA containing BUDR is sufficiently different in its structure that regulatory controls are abortive and ineffective (Wilt and Anderson, 1972). There are many versions of this type of model. For instance, if there are regulatory elements adjacent to structural genes, such as proposed by Britten and Davidson (1969), the presence of BUDR in these elements could explain why some functions are affected more than others. A prediction of this type of hypothesis is that occasionally a cell function should appear that is not normally present e.g., interference with regulation might stimulate synthesis of enzymes under negative control. Koyama and Ono (1971) have found a 7-fold elevation of alkaline phosphatase in B-6 cells cultured in BUDR; this is consistent with the prediction, though not sufficient to prove it. In other versions of this type of model, it has been suggested that the effect of BUDR may be on the structure of chromatin,

as opposed to just DNA (Miura and Wilt, 1971). One known effect of BUDR is production of chromosomal abnormalities. It could be that the presence of large amounts of BUDR affects binding of the chromatin proteins, thereby perturbing the way in which some regulatory events may take place.

All of the above mentioned hypotheses or models to explain the mechanism whereby BUDR exerts its effects on differentiation have some merit. However, at the present time, neither one is generally accepted, and the mode of action of BUDR on differentiated cell systems still remains a mystery.

Dimethyl sulphoxide (DMSO) DMSO is an extraordinary organic solvent with a wide range of biological activities (Leake, 1967). One of its most interesting activities is its effect on the differentiation of certain cell types. In 1971, Friend et al. reported that cells (Friend Leukemic Cells) of a cloned line of murine virus-induced erythroleukemia, maintained in cell culture, were stimulated to differentiate along the erythroid pathway by DMSO at concentrations that did not inhibit cell growth. Differentiation was demonstrated by the induction of heme and hemoglobin synthesis. Exposure of the cells to DMSO for one hour followed by transfer to DMSO-free media was sufficient to "trigger" the synthesis of hemoglobin. Control cells grown in the absence of this agent showed no signs of differentiation. The effect may be reversed by the removal of the DMSO from the growth media. Other investigators (Ross et al., 1972; Preisler et al., 1973; Singer et al., 1974; Gilmour et al., 1974) have reported similar findings using other cultured leukemia cells.

The mechanism of action of DMSO in stimulating differentiation of the

erythroleukemic cells is yet to be understood. Evidence that it affects the cell membrane comes from the studies of Weiner et al. (1972) who found the penetrative ability of DMSO might be due to some alteration of protein structure as a result of dehydration of the biomembrane. Phospholipids protected the protein from attack by DMSO. That DMSO exerts an effect on the regulatory role of lipids for RNA synthesis in mammalian cells has been suggested by Lezius and Müller-Lorensen (1974) who found that DMSO both stabilizes and stimulates the activity of RNA polymerase A, an enzyme stiumlated by neutral phospholipids, by changing the template specificity from double stranded in favor of single stranded DNA. Other activities of DMSO which may be significant are its ability to stimulate microsomal Ca^{++} uptake and to inhibit Na^{+}-, K^{+}-dependent adenosine triphosphatase (Burgess et al., 1969). Friend et al. (1974) also observed that DMSO stimulates adenyl cyclase.

MATERIALS AND METHODS

Cell Lines

Two cell lines were used in this study:

1. GF9. GF9 is a mouse white blood cell line deficient in the enzyme, hypoxanthine-guanine phosphoribosyl transferase ($HGPRT^-$). GF9 was derived by mutagenesis from a population of L5178Y cells (subclone 43). The L5178Y lymphocytic parent was first isolated from a leukemic mouse by Law, and later grown in vitro by Fischer (1958). GF9 cells are lymphocytic in appearance, near diploid, and neoplastic. These cells are grown in Fischer's medium (Table 2) supplemented with 10 percent (v/v) heat-inactivated horse serum. They are incubated at 37^oC in a five percent carbon dioxide, 95 percent air atmosphere. Grown under these conditions, GF9 cultures have a doubling time of 10-11 hours (Figure 4).
2. Intestine 407 (Embryonic Intestine: Human). Intestine 407 was isolated by Henle and Deinhardt (1957) from the jejunum and ileum of a two month old human embryo. These cells are grown attached to the surface of a culture flask (Falcon Plastics) in Eagle's basal medium with Hank's salts (Table 3) supplemented with 15 percent (v/v) heat-inactivated fetal calf serum. They are incubated at 37^o C in an atmosphere of five percent carbon dioxide. Under these conditions, Intestine 407 has a doubling time of approximately 18 hours. The cells are epithelial-like in appearance and are aneuploid. The cell line was obtained from the American Type Culture Collection, Rockfield, Maryland.

Table 2. Components of Fischer's Medium

Component	Mg/Liter
NaCl	8000.0
$MgCl_2 \cdot 6H_2O$	100.0
$NaH_2PO_4 \cdot 6H_2O$	69.0
$Na_2HPO_4 \cdot 7H_2O$	113.0
KCl	400.0
$CaCl_2 \cdot 2H_2O$	91.0
Glucose	1000.0
L-Arginine	15.0
L-Cystine	20.0
L-Glutamine	204.0
L-Histidine HCl	60.0
L-Isoleucine	75.0
L-Leucine	30.0
L-Lysine HCl	50.0
L-Methionine	100.0
L-Phenylalanine	60.0
L-Threonine	30.0
L-Tryptophan	10.0
L-Tyrosine	60.0
L-Valine	70.0
L-Asparagine	10.0
L-Serine	15.0
Biotin	0.01
Choline Cl	1.50
Folic Acid	10.00
i-Inositol	1.50
Nicotinamide	0.50
Ca-pantothenate	0.50
Pyridoxal HCl	0.50
Riboflavin	0.50
Thiamine HCl	1.00
Phenol Red	5.00
$NaHCO_3$	1125.00

Figure 4. Growth curve of GF9 cells in Fischer's Medium supplemented with 15 percent horse serum

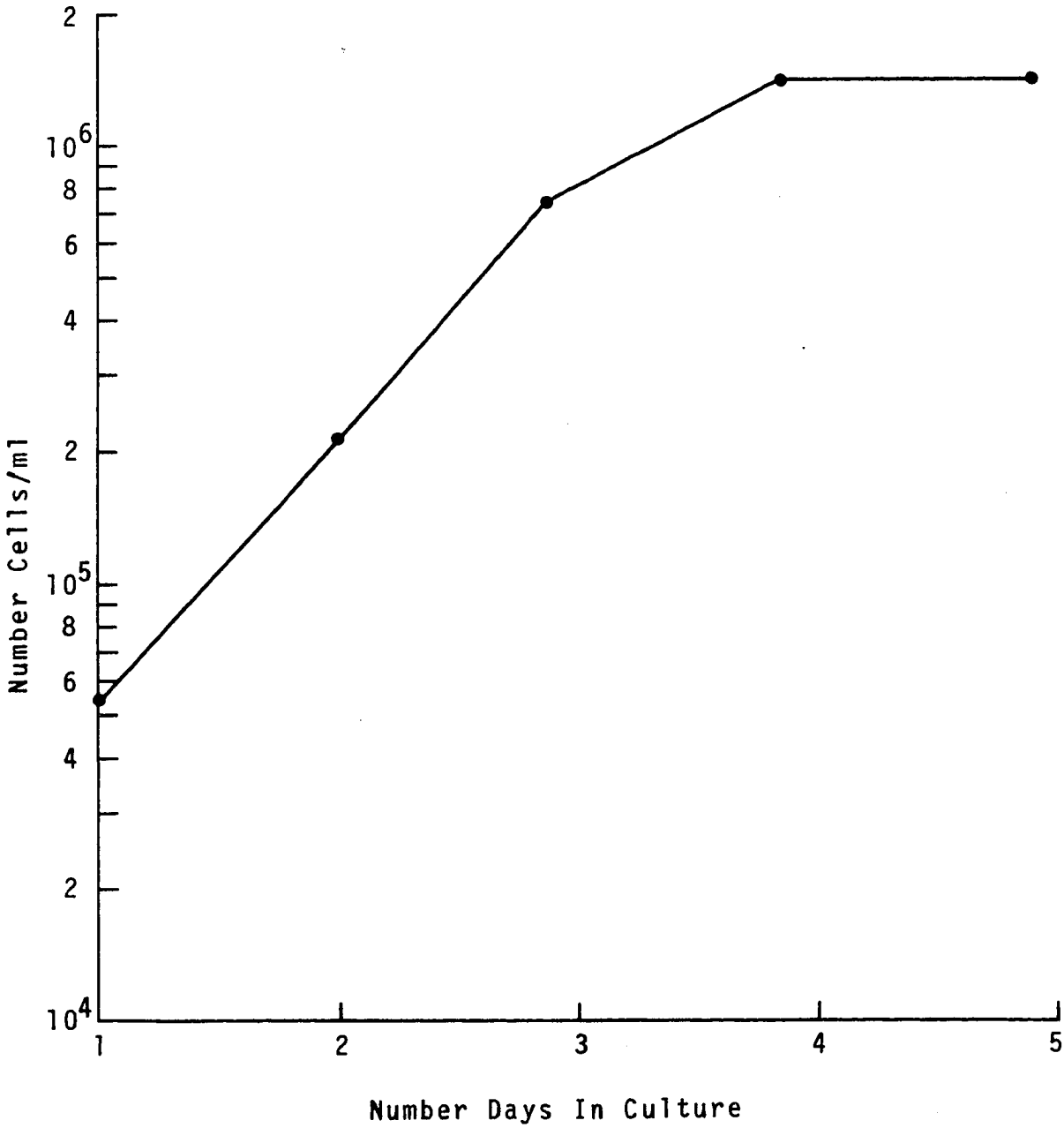

2
10^6
8
6
4
2
10^5
8
6
4
2
10^4
Number Cells/ml
1
2
3
4
5
Number Days In Culture

Table 3. Components of Eagle's Basal Medium with Hanks' salts

Component	Mg/Liter
$CaCl_2$	140.00
KCl	400.00
KH_2PO_4	60.00
$MgCl_2 \cdot 6H_2O$	100.00
$MgSO_4 \cdot 7H_2O$	100.00
NaCl	8000.00
$NaHCO_3$	350.00
$Na_2HPO_4 \cdot 2H_2O$	60.00
Glucose	1000.00
L-Arginine	17.40
L-Cystine	12.00
L-Glutamine	292.00
L-Histidine	8.00
L-Isoleucine	26.00
L-Leucine	26.00
L-Lysine	29.20
L-Methionine	7.50
L-Phenylalanine	16.50
L-Threonine	24.00
L-Tryptophane	4.00
L-Tyrosine	18.00
Biotin	1.00
D-Ca-pantothenate	1.00
Choline Chloride	1.00
Folic Acid	1.00
i-Inositol	1.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	1.00
Thiamine HCl	1.00

Media and Chemicals

The media and sera used for maintenance of the cell lines were obtained from the Grand Island Biological Supply Company, Grand Island, New York. Ethylmethane sulfonate, N-methyl-N-nitro-N-nitrosoguanidine, bromodeoxyuridine and isopropyl thioglactoside were obtained from the Sigma Chemical Company. Lactose was secured from Mann Research. Dimethyl sulphoxide was purchased from Fischer Scientific and colcemid was obtained from CIBA Pharmaceuticals.

Cell Counts

Determinations of cell number were made using a standard cell counter (Model ZBI, Coulter Electronics) and by hemacytometer. The cell counter is used primarily for the enumeration of cells grown in suspension culture. The hemacytometer is used for counting attached cell lines and for making viable counts on all cells.

Viable cell counts involve the use of the vital stain, trypan blue, which is mixed 1:1 with cells in growth medium. Nonviable cells incorporate the stain and become blue while viable cells do not.

Treatment With Mutagen

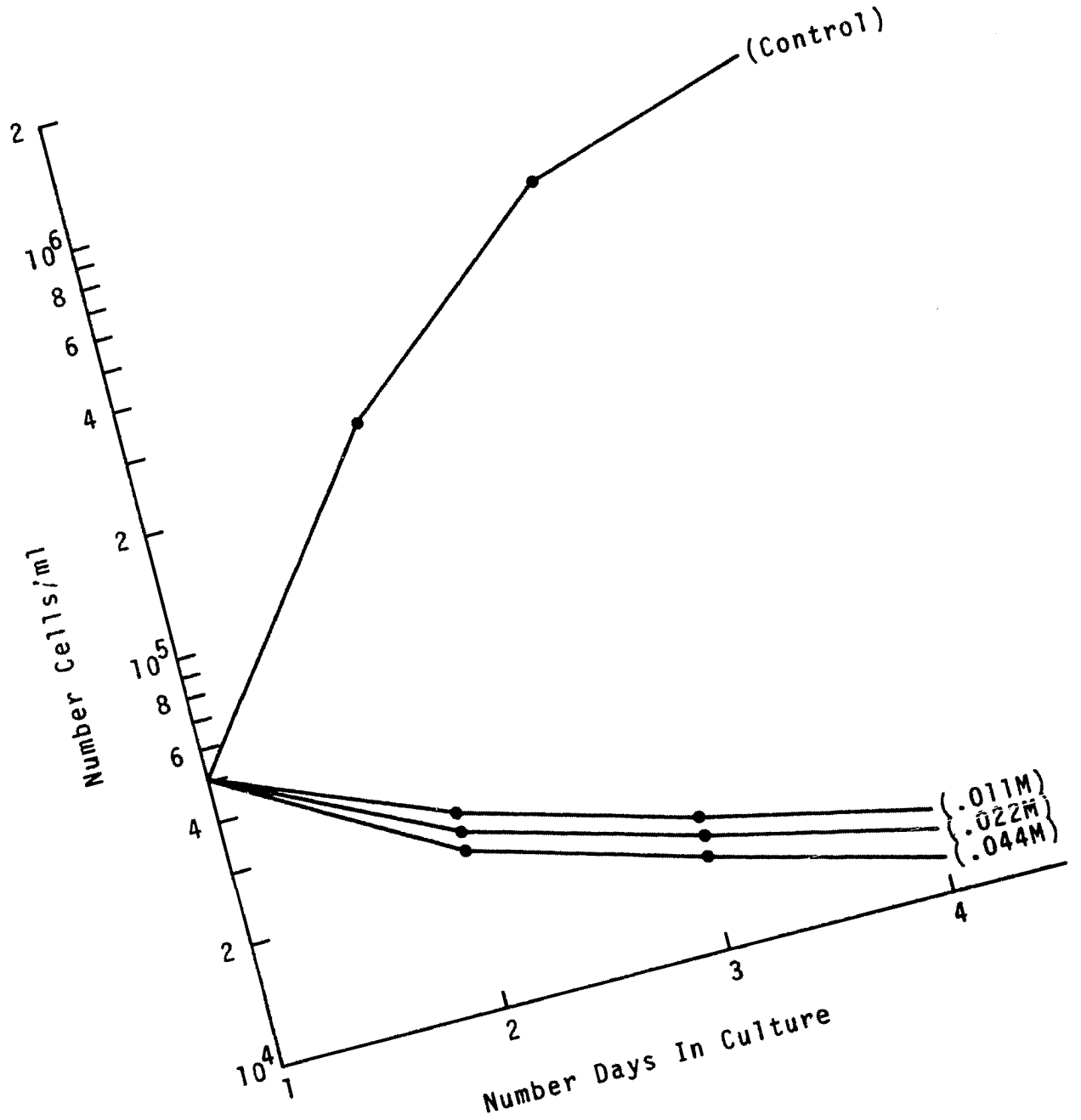
GF9 cells growing exponentially (10^5 cells/ml) were treated with various levels of ethylmethane sulfonate (EMS) or N-methyl-N-nitro-N-nitrosoguanidine (NG) at 37^{o} in Fischer's medium supplemented with 10 percent horse serum. Cells were exposed to the mutagen for two hours. Then the cells were centrifuged, washed once in mutagen-free media and resuspended in fresh media without mutagen. The cells were then either

cloned immediately in soft agar medium to determine cell survival or were grown in suspension for 48 hours in order to allow expression of the mutation before exposure to the selective medium. Survivors of the mutagen treatment were measured by cloning 100 cells per 15 x 150 mm test tube in soft agar medium at each mutagen concentration. Since each single cell is capable of giving rise to a colony of cells, the percentage of cells that survived the treatment was determined simply by counting the number of colonies obtained. EMS and NG were stored frozen (-20^{o}) at 0.001 g/ml in phosphate buffered saline (pH 7.2) and small aliquots were thawed just before use.

Cloning in Soft Agar

In these experiments GF9 was cloned using the method of Chu and Fischer (1968) developed for cloning cells in soft agar gel suspension. Three milliliters of Fischer's medium containing 0.2 percent agar (Noble agar, Difco Lab.) and 15 percent horse serum were added to 15 x 150 mm test tubes. These tubes were maintained at 45^{o} to prevent solidification of the agar and then before the addition of cells were removed to a 37^{o} water bath. Fifty to a hundred cells in two milliliters of Fischer's medium plus 15 percent horse serum were added to each tube to give a final concentration of 0.12 percent agar. After gentle mixing of the cells and agar, the tubes were placed upright in an ice bath for 2.5 minutes and then placed in tissue culture racks for incubation at 37^{o}. To minimize breakdown of the agar gel, the racks were placed on foam rubber mats which insulated the tubes from vibration during the incubation. Using this pro-

Figure 5. Growth curves of GF9 cells in Fischer's Medium containing 15 percent horse serum (control) and in Fischer's Medium containing different concentrations of lactose and 15 percent dialyzed horse serum


(Control)
2
10^6
8
6
4
2
10^5
8
6
4
2
10^4
Number Cells/ml
(.011M)
(.022M)
(.044M)
1
2
3
4
Number Days In Culture

cedure, GF9 has a cloning efficiency of 80-90 percent.

Colonies ranging from 0.5-1.0 mm in diameter were visible seven to eight days later. An average colony contained approximately 10^5 viable (trypan blue excluding) cells. Colony counts were made using a Quebec darkfield colony counter (American Optical Company). Mature colonies were isolated by suction with a pasteur pipette and dispersed in 0.5 ml medium in a test tube where the cells resumed growth in suspension culture.

Selection For Lactose (lac^+) Mutants

By formulation Fischer's medium (Table 2) contains 0.0055 M (1g/liter) glucose. In the selective medium, all glucose is replaced by 0.011 M (3.76 g/liter) lactose. Lactose is a disaccharide consisting of one molecule of glucose and one molecule of galactose. On hydrolysis these components are released in equal quantities. Thus, to insure that the original glucose concentration may be restored, twice as much lactose is used in the selective medium.

The complete selective medium contains Fischer's medium modified by lactose plus 15 percent dialyzed horse serum. In order to remove glucose the horse serum was dialyzed for 20 hours against three volume changes of 0.85 percent saline and tested for the presence of glucose by the "glucostat" (Worthington Biochemicals), an enzymatic reaction for the detection of glucose, and was found to be free of this sugar.

The parental GF9 cell is unable to utilize lactose as a carbon source (Figure 5). Thus, only mutant cells would be expected to survive in selective medium where lactose is provided as the sole carbon source. Selection for lactose utilizing (lac^+) mutants was performed by plating

4.0×10^5 mutagen treated viable cells per test tube in the selective medium containing soft agar. Variant colonies were visible three weeks after plating and were isolated at that time in 0.5 ml selective medium or 0.5 ml nonselective medium in order to obtain suspension cultures. Colonies isolated in nonselective medium (Fischer's medium containing glucose plus 15 percent nondialyzed horse serum) were tested on the selective medium when a sufficient volume of cells were obtained.

Enzyme Induction Experiments

Two agents were utilized as potential inducers of the lactase enzyme in the mammalian cell lines: lactose and isopropyl B-D-thiogalactopyranoside (IPTG). Both of these compounds are inducers of beta galactosidase in bacteria (Jacob and Monod, 1961). The induction procedure was as follows:

GF9 and mutant colonies

The cells were allowed to grow to a density of 5.0×10^5 cells/ml in the nonselective medium. They were then centrifuged, washed once in sugar-free medium and resuspended in Fischer's medium containing various levels of lactose and 15 percent dialyzed horse serum or in the same medium containing different levels of IPTG plus 15 percent dialyzed horse serum. The cells were incubated in this medium for a period of 12 hours (the cellular glucose is depleted in these cells in approximately six hours). Thereafter, the cells were harvested and assayed for lactase activity.

Intestine 407

The cells were allowed to grow until a confluent monolayer of cells was obtained. The medium was then removed and the monolayer washed once with sugar-free medium. Eagle's basal medium without sugar plus 15 percent dialyzed fetal calf serum was then added to the cells and the cultures were incubated in this medium for 12 hours to insure depletion of glucose in the cells. At the end of this incubation period, different concentrations of lactose or IPTG were added to the cells and incubation continued for another 12 hours. Thereafter, the cells were harvested and assayed for lactase activity.

Treatment of Cells with BUDR and DMSO

BUDR and DMSO were added to the growth medium in the concentrations cited in the Results section. For the dose response curves, replicate cultures were incubated at cell concentrations of 5.0×10^4 cells/ml in medium with these chemicals. Viable cell counts were made daily.

The concentration of BUDR and DMSO which did not appreciably affect cell growth was then used with medium containing lactose in an attempt to induce lactase production and subsequent growth of GF9 cells in medium containing lactose as the sole carbon source.

Determination of Enzyme Activity

Lactase activity was assayed by a modification of the method described by Dahlqvist (1964). Cells growing exponentially ($5.0\text{-}6.0 \times 10^5$ cells/ml) were harvested by centrifugation, washed once in a balanced salt solution and resuspended in distilled water at $4.0\text{-}7.0 \times 10^7$

cells/ml. The cells were then lysed by rapid freezing and thawing until a homogenous extract was obtained (four to five freeze-thaw cycles). This preparation was then centrifuged at 20,000 x g for 10 minutes and the supernatant used for the assay. The assay procedure was carried out in the following manner: one milliliter of the supernatant was mixed with 0.1 ml of the substrate (0.056 M lactose) in a small test tube and incubated in a 37^{0} water bath for one hour. The enzymatic reaction was stopped by immersing the tube in boiling water for two minutes. For determination of the amount of glucose present, 0.5 ml of the above reaction mixture was mixed with 3 ml of tris glucose oxidase reagent (Worthington Biochemicals) and incubated at 37^{0} for one hour. The light absorption was then measured in a Beckman DU-2 spectrophotometer at 420 mu using one centimeter cuvettes.

An extract prepared from fetal mice small intestine which is known to contain lactase activity was used as a control for the assay.

Establishment of a Primary Cell Line from Fetal Mouse Intestine

Pregnant mice (Mus musculus) were obtained from Dr. Willard Hollander, Genetics Laboratory, Iowa State University. On the 18th day of gestation, the mice were sacrificed and the fetuses were removed. The entire small intestine was removed from each fetus and washed several times in Hank's balanced salt solution containing 20,000 units of penicillin and 20,000 units of streptomycin (GIBCO). The intestinal tissue was then cut into small pieces and incubated in a 0.25 percent trypsin-EDTA (0.5 g trypsin: 0.2 g EDTA/liter) solution for 15 minutes to disperse the tissue. The trypsin activity was stopped by the addition of an equal volume of horse

serum. The cells were then filtered through a double layer of cheese cloth to remove chunks of tissue that had not been dispersed. The single cells were centrifuged at 1500 r.p.m. for 10 minutes and resuspended in Eagle's basal medium supplemented with 15 percent horse serum. The cells were placed in 25 cm^2 culture flasks (Falcon Plastics) at a concentration of 10^6 cells/flask and incubated at 37^o.

Infection of Fetal Mouse Small Intestinal Cells with SV-40 Virus

The SV-40 virus was isolated from baboon kidney cells grown in culture. It was obtained from the Southwest Foundation for Research and Education, San Antonio, Texas. The titer of the virus was $10^{6.3}TCID_{50}$ (tissue culture infectious dose for 50 percent response) per 0.1 ml. The 50 percent infectious dose is the smallest volume of virus capable of producing a response in 50 percent of the susceptible cells.

Flasks of cells were exposed to 0.5 and 0.25 ml of virus for periods of four or 24 hours. The cultures were then washed and fresh medium was added; the medium was changed daily.

Chromosome Analysis

The mitotic chromosomes of GF9 and variant colonies were prepared in the following way: cells growing exponentially were arrested in metaphase by adding colcemid at a final concentration of 0.2 ug/ml to the culture medium. The cells were incubated in the presence of this drug for a period of four to six hours at 37^o. Following the incubation period the cells were pelleted by centrifugation at 1200 r.p.m. for six minutes. The pellet was resuspended in 3 ml hypotonic KCl (0.75 M).

This agent induces swelling and was left on the cells for 8 minutes. The treated cells were then centrifuged for 6 minutes at 600 r.p.m. All but 0.5 ml of the supernatant was removed by aspiration and the cells were gently resuspended in the remaining supernatant and fixed in Carnoy's fixative (one part glacial acetic acid:three parts absolute methanol) at $4^{o}C$ for 10 minutes. The cells were centrifuged and all of the supernatant was removed by aspiration. The pellet was resuspended in Carnoy's fixative and fixed for another 10 minute period at $4^{o}C$. At the end of this fixation period the cells were collected by centrifugation and all but 0.25 ml of the supernatant was removed by aspiration. The cells were resuspended in the residue by gentle agitation with a pasteur pipette. Two to three drops of the cell suspension was then placed on a slide (stored in cold distilled water) and quickly blown to spread as widely as possible. The slide was allowed to air dry for 15 minutes and was then stained with aceto orcein stain (orcein obtained from Gurr, Biomedical Specialities).

RESULTS

Sensitivity of GF9 Cells to EMS and NG

The survival of GF9 cells after treatment with various concentrations of the mutagens, EMS and NG, is shown in Figure 6. Both curves are multihit curves (Kao and Puck, 1969, 1971) which is an indication that some threshold process must be exceeded before cell killing occurs in linear proportion to the dose of mutagen. In mutagenesis experiments, that concentration of mutagen which gave a cell survival of approximately 20 percent was used. For EMS this was 1.3×10^{-2} M and for NG, 10^{-6} M. Similar values were obtained in EMS and NG survival experiments with the related cell strain L5178Y (Summers, 1973).

Induction and Isolation of Lactose (lac^+) Variants

Tables 4 and 5 summarize the results of mutagenesis experiments with EMS and NG respectively. An average of 9.69 variant colonies per 10^5 surviving cells was obtained when the parental cells were treated with EMS and plated in selective medium in which lactose was the sole carbon source provided. Treatment with NG yielded an average of 1.35 variants per 10^5 surviving cells. Colonies recovered from the selective medium were either subcultured directly in liquid selective medium (to test their ability to utilize lactose in suspension culture) or, they were first isolated in nonselective medium and then tested in medium containing lactose and 15 percent dialyzed horse serum. As the last column in Tables 4 and 5 indicates, none of the colonies tested proved to be able to utilize lactose in suspension culture. It would be expected that true

Figure 6. Survival of GF9 cells after treatment with various concentrations of EMS for 2 hours (A) or NG for 2 hours (B). For each point 16 replicate tubes containing 100 cells/tube is plotted.

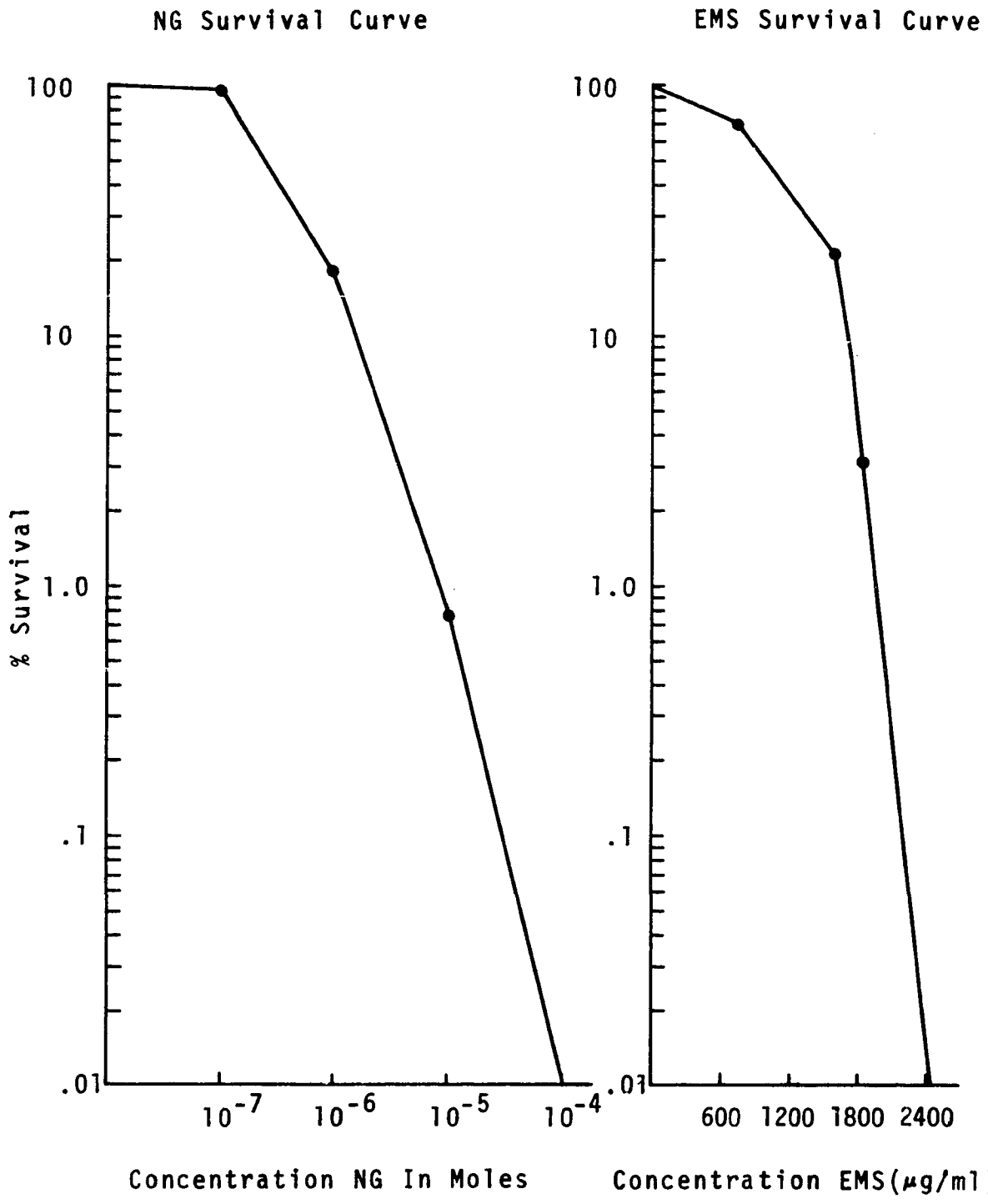

NG Survival Curve
EMS Survival Curve
100
10
1.0
.1
.01
% Survival
10^{-7}
10^{-6}
10^{-5}
10^{-4}
600
1200
1800
2400
Concentration NG In Moles
Concentration EMS(μg/ml)

Table 4. Summary of EMS mutagenesis experiments[a]

Number Viable Cells Plated	Percent of Plated Cells Surviving[b]	Number of Variant Colonies Recovered[c]	Frequency of Variant Colonies in Population of Surviving Cells ($\times 10^{-5}$)	Number of Colonies Tested Able to Utilize Lactose[d]
8.56×10^7	2.70	121	5.26	0
14.00×10^7	1.40	452	6.36	0
6.27×10^7	2.60	182	11.37	0
6.64×10^7	3.00	304	11.60	0
7.64×10^7	3.37	218	8.48	0
6.64×10^7	5.35	278	7.94	0
7.68×10^7	3.46	292	11.00	0
13.40×10^7	2.98	357	8.94	0
8.00×10^7	3.00	227	9.45	0
7.50×10^7	2.65	187	9.44	0
6.40×10^7	3.15	205	10.20	0
10.00×10^7	3.00	327	10.91	0
10.50×10^7	2.53	397	15.10	0
113.23×10^7	-	3547	-	0
Average values:	3.01	-	9.69×10^{-5}	-
Cumulative Control[e]:				
1.99×10^8	8.30	2.00	.012	0

[a]EMS concentration + 1.3×10^{-2} M.

[b]Cells were plated in nonselective medium supplemented with 15 percent dialyzed horse serum.

[c]Cells were plated in selective medium supplemented with 15 percent dialyzed horse serum.

[d]In each experiment 100 colonies were tested for growth in liquid selective medium.

[e]Cells were not exposed to mutagen but were plated in selective medium.

Table 5. Summary of NG mutagenesis experiments [a]

Number Viable Cells Plated	Percent of Plated Cells Surviving[b]	Number of Variant Colonies Recovered[c]	Frequency of Variant Colonies in Population of Surviving Cells ($\times 10^{-5}$)	Number of Colonies Tested Able to Utilize Lactose[d]
8.0×10^7	2.93	24	1.00	0
7.6×10^7	2.50	26	1.30	0
6.0×10^7	3.18	24	1.26	0
7.36×10^7	2.75	21	1.03	0
4.84×10^7	2.62	14	1.16	0
7.68×10^7	2.75	51	2.37	0
41.48×10^7	-	160	-	0
Average Values:	2.78	-	1.35×10^{-5}	-
Cumulative Control[e]:				
1.99×10^8	8.30	2.0	.012	-

[a] NG concentration = 10^{-6} M.

[b] Cells were plated in nonselective medium supplemented with 15 percent dialyzed horse serum.

[c] Cells were plated in selective medium supplemented with 15 percent dialyzed horse serum.

[d] All variant colonies in each experiment were tested for growth in liquid selective medium.

[e] Cells were not exposed to mutagen, but were plated in selective medium.

lactose-utilizing mutants would grow in suspension culture as well as agar culture when lactose is the only carbon source provided. Therefore, the colonies are referred to as "lactose selection variants".

Control experiments were carried out to determine whether these variants occurred spontaneously or if they arose as the result of mutagenic action. Untreated GF9 cells were cloned in selective medium at a concentration of 4.0×10^5 viable cells per tube. The results of these trials are given in Table 6. In these tests two colonies were recovered per 1.99×10^8 viable cells plated. However, this number is insignificant when compared with the number of colonies obtained after treatment with the mutagens, EMS and NG. A total of 3547 colonies were recovered per 1.13×10^9 cells plated following EMS treatment (Table 4); and 160 colonies per 4.14×10^8 cells plated were obtained after treatment with NG (Table 5).

Studies of the Origin of "Lactose Selection Variants"

Since the mutagen-induced variants did not prove to have the phenotype expected to be associated with lac^+ mutants, i.e., the ability to utilize lactose in suspension culture, further attempts were made to characterize the events causing the appearance of these colonies. Four alternative situations were explored.

The first alternative

The first situation considered was that the "lactose selection variants" might have resulted from growth on trace amounts of residual glucose in the selective medium. This possibility was examined by

Table 6. Experiments to detect spontaneous mutational events which yielded "lactose selection variants"

Number Viable Cells Plated	Percent of Plated Cells Surviving in Nonselective Medium[a]	Number of Variant Colonies Recovered in Selective Medium[b]	Frequency of Variant Colonies in Population of Surviving Cells ($\times 10^{-5}$)
2.0×10^7	6.88	0.0	0.000
4.9×10^7	8.73	0.0	0.000
6.0×10^7	7.48	2.0	.012
7.0×10^7	10.03	0.0	0.000
19.9×10^7	-	2.0	-
Average Values:	8.28	-	$.003 \times 10^{-5}$

[a]Fischer's Medium plus 15 percent dialyzed horse serum.

[b]Fischer's Medium containing lactose plus 15 percent dialyzed horse serum.

conducting sugar analyses of all components of the selective medium. Since the parental cells can utilize galactose as well as glucose as a carbon source, the selective medium components were tested for both glucose and galactose using the "glucostat" and the "galactostat" (Worthington Biochemicals). These are prepared reagents for the quantitative and colorimetric determination of glucose and galactose respectively. The "glucostat" contains glucose oxidase; and the "galactostat" contains galactose oxidase. For a complete explanation of these tests and the assay procedure, the reader is referred to the Worthington Enzyme Manual (Worthington Biochemical Corporation, 1972).

The standard curves for the "glucostat" and the "galactostat" are given in Figures 7 and 8 respectively. The average optical density (O.D.) reading for the "glucostat" reagent blank was 0.015. It was assumed that any O.D. value greater than or equal to 0.015 would be significant. A 0.015 O.D. value gives a glucose concentration of 10 mg/100 ml or 5.0×10^{-4} M. Therefore, if glucose were present in a sample at a concentration greater than or equal to 5.0×10^{-4} M it would have been detected.

The average O.D. reading for the reagent blank in the "galactostat" was 0.045. It was supposed that any O.D. value greater than or equal to 0.045 would be significant. This absorbance at 425 mu gives a galactose concentration of 15 ug/0.5 ml or 2.0×10^{-4} M. If components of the selective medium had contained galactose at an amount greater than or equal to 2.0×10^{-4} M, it would have been ascertained.

The results of these tests are given in Table 7. It was described

Figure 7. Glucose standard curve for the "glucostat" reagent

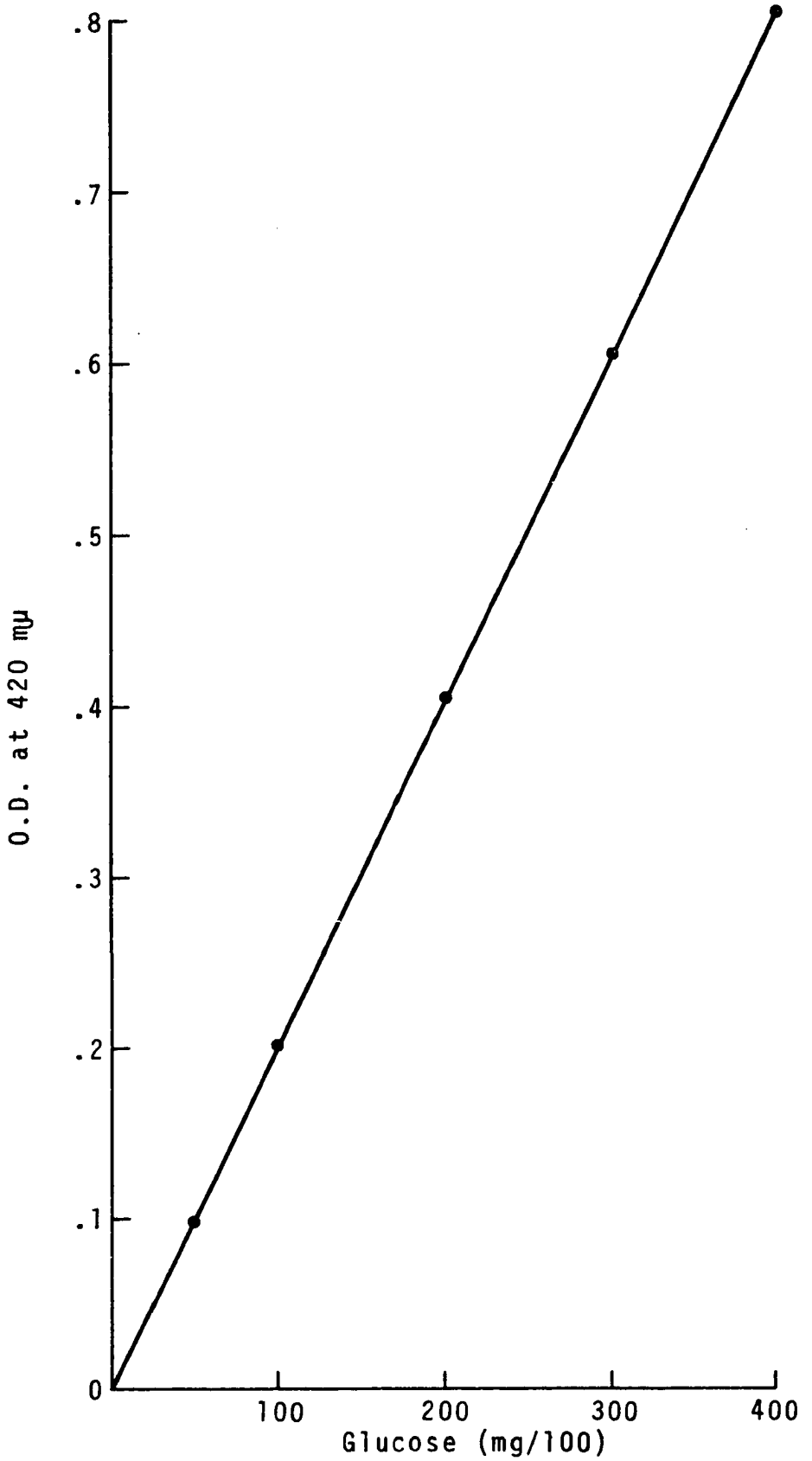

.8
.7
.6
.5
.4
.3
.2
.1
0
O.D. at 420 mμ
100
200
300
400
Glucose (mg/100)

Figure 8. Galactose standard curve for the "galactostat" reagent

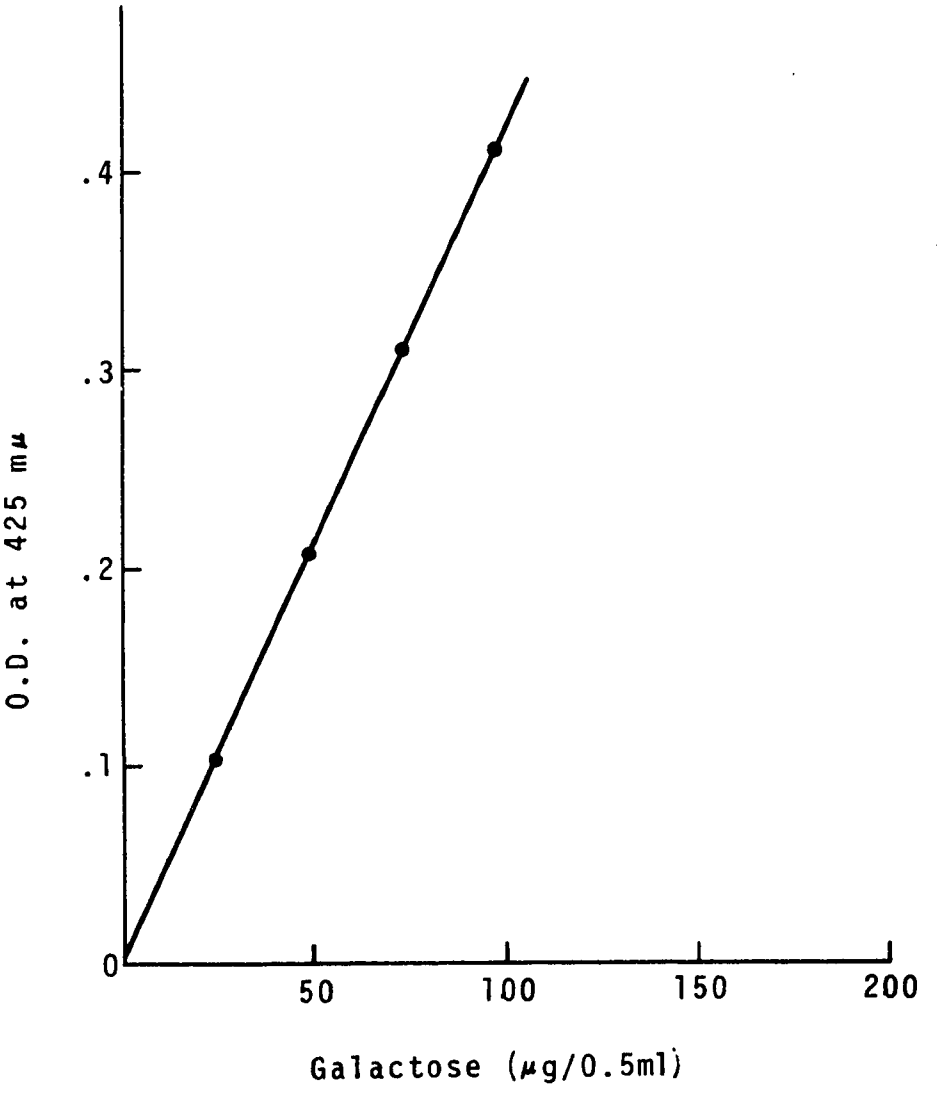

O.D. at 425 mμ
.4
.3
.2
.1
0
50
100
150
200
Galactose (μg/0.5ml)

Table 7. Tests for residual glucose and galactose in selective medium components

Component	Amount of Glucose (Moles/Liter)	Amount of Galactose (Moles/Liter)
All chemicals in Fischer's medium excluding glucose[a]	$< 5.0 \times 10^{-4}$	$< 2.0 \times 10^{-4}$
Noble Agar (0.12%)	$< 5.0 \times 10^{-4}$	$< 2.0 \times 10^{-4}$
Lactose:		
0.011 M	$< 5.0 \times 10^{-4}$ [b]	$< 2.0 \times 10^{-4}$
0.044 M	$< 2.0 \times 10^{-3}$ [b]	$< 4.0 \times 10^{-4}$ [c]
0.500 M	$< 2.5 \times 10^{-2}$ [b]	$< 1.0 \times 10^{-3}$ [c]
1.000 M	$< 5.0 \times 10^{-2}$	$< 2.0 \times 10^{-2}$ [c]
Dialyzed horse serum	$< 5.0 \times 10^{-4}$	$< 2.0 \times 10^{-4}$
Controls:		
Fischer's Medium (Standard formula[a])	5.5×10^{-3}	-
1.0 percent Galactose solution	-	2.8×10^{-2}

[a]See Table 4.

[b]Interpolated values for amount of glucose in sample. See text for explanation.

[c]Interpolated values for amount of galactose in sample. See text for explanation.

above that the "glucostat" was insensitive to levels of glucose less than 5.0×10^{-4} M; and the "galactostat" insensitive to levels of galactose less than 2×10^{-4} M. Hence, it is possible that each component tested was contaminated with one of these sugars at an amount lower than the level of detection of these assays. Accordingly, these values were used in Table 7 to indicate limits for the amount of glucose or galactose that might have been present in each selective medium component. However, significant O.D. readings, i.e., O.D. readings above that of the control blank, were not obtained for any of the components tested using the prepared reagents.

Fischer's medium lacking glucose was specially prepared for use in this study by the Grand Island Biological Supply Company, Grand Island, New York. This medium consists of only salts, amino acids, and vitamins. Therefore it is very unlikely that it contained any sugar. It is also equally unlikely that the Noble Agar contained any free sugar (personal communication, Difco Lab. technical advisor).

The lactose which was used in the selective medium at a concentration of 0.011 M was checked for glucose and galactose impurities at concentrations of 0.044 M, 0.5 M, and 1.0 M. These amounts were 4, 50, and 100 times the normal amount used respectively. If 0.011 M lactose contains less than 5.0×10^{-4} M glucose or less than 2×10^{-4} M galactose, increasing the amount of lactose would increase the probability of the sugar being detected. For example, if glucose were present at less than 5.0×10^{-4} M in a 0.011 M lactose solution, the amount in a 0.044 M solution would have been less than 2.0×10^{-3} M; in a 0.5 M solution less than

2.5×10^{-2} M; and in a 1.0 M solution less than 5.0×10^{-2} M. These values would be well within the detectable range of the "glucostat". The same argument holds for galactose (Table 7). Positive results were not obtained when the above concentrations of lactose were tested. Therefore, the lactose used in the selective medium did not contain glucose or galactose impurities.

Definite conclusions regarding the presence or absence of trace amounts of glucose or galactose in the dialyzed horse serum could not be drawn using the above chemical tests. However, dialysis for 20 hours against three volume changes of 0.85 percent saline should have been sufficient to remove all sugar. Furthermore, data presented in Figures 9 and 10 and in Table 8 show that if the dialyzed horse serum did contain trace amounts of glucose or galactose, these amounts were evidently inadequate to support cell growth in the absence of sufficient quantities of added, metabolizable sugar. Therefore the dialyzed horse serum was not responsible for the appearance of colonies in the selective medium.

In relation to the first possibility, that the appearance of the "lactose selection variants" was due to growth on residual glucose or galactose in the selective medium, it was assumed that the colonies might have been mutagen-induced, stable variants capable of growing in medium containing very low levels of glucose or galactose not detectable by the tests described above. Since glucose is the standard sugar in culture medium, this theory was investigated by performing growth experiments on parental cells in medium containing different concentrations of glucose and dialyzed horse serum. Growth curves of untreated GF9 cells on various

levels of added glucose are shown in Figure 9. These growth studies were carried out on the assumption that the dialyzed horse serum was probably free of glucose. However, as indicated above, the serum might possibly have contained some glucose (less than 5.0×10^{-4} M). Thus the glucose concentrations stated in Figure 9 may need to be adjusted. Note that the lowest concentration of added glucose at which there is no appreciable growth is 5.5×10^{-6} M. If the isolated colonies were indeed stable variants capable of growing in low levels of glucose, they should have been able to grow at this concentration of glucose which would not support growth of the parental cells.

Twenty variant colonies were tested to determine if they could proliferate in medium containing this amount of glucose. Typical growth curves for 20 of the variants are shown in Figure 10. This level of glucose did not support growth of the "lactose selection variants". Therefore, it is not reasonable to conclude that these were stable variants that appeared in the selective medium because of a new, mutagen-induced capability for growth on low levels of glucose.

The second alternative

The second alternative tested was that the variants might have resulted from cross-feeding between cells cloned as clumps rather than as individual cells. Cross-feeding is an event in which some cells of a group supply nutrients that promote the growth and formation of colonies by surrounding cells. In this case, cross-feeding would mean that one or two clumped cells would have been able to provide enough carbon for cell

Figure 9. Growth curves of GF9 in Fischer's Medium containing different concentrations of glucose plus 15 percent dialyzed horse serum. RHS, nondialyzed horse serum; DHS, dialyzed horse serum; Glu., glucose.

1. Control-.0055M Glucose + 15% RHS
2. .0055M Glu. + 15% DHS
3. .00055M Glu. + 15% DHS
4. .000055M Glu. + 15% DHS
5. .0000055M Glu. + 15% DHS

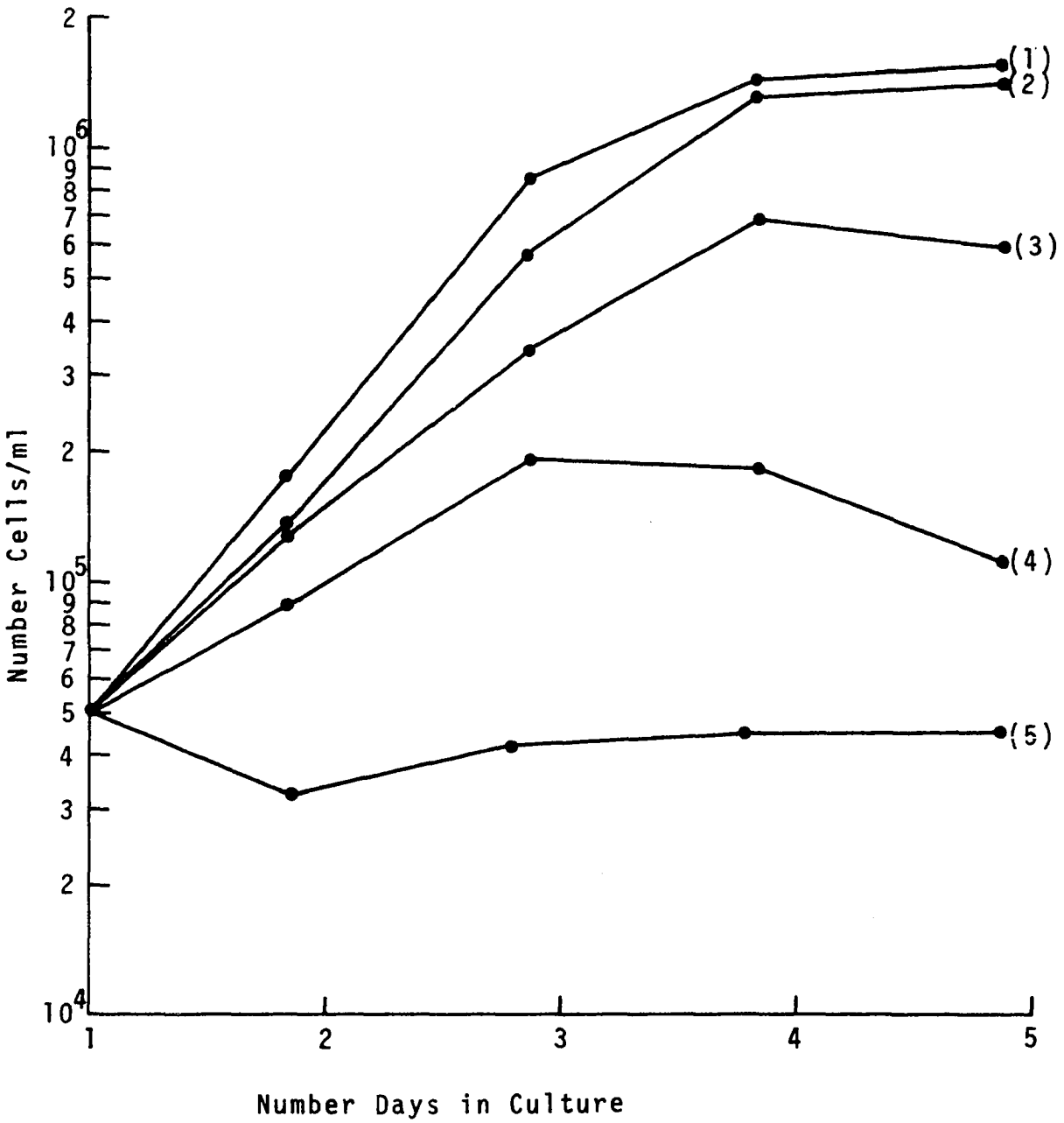
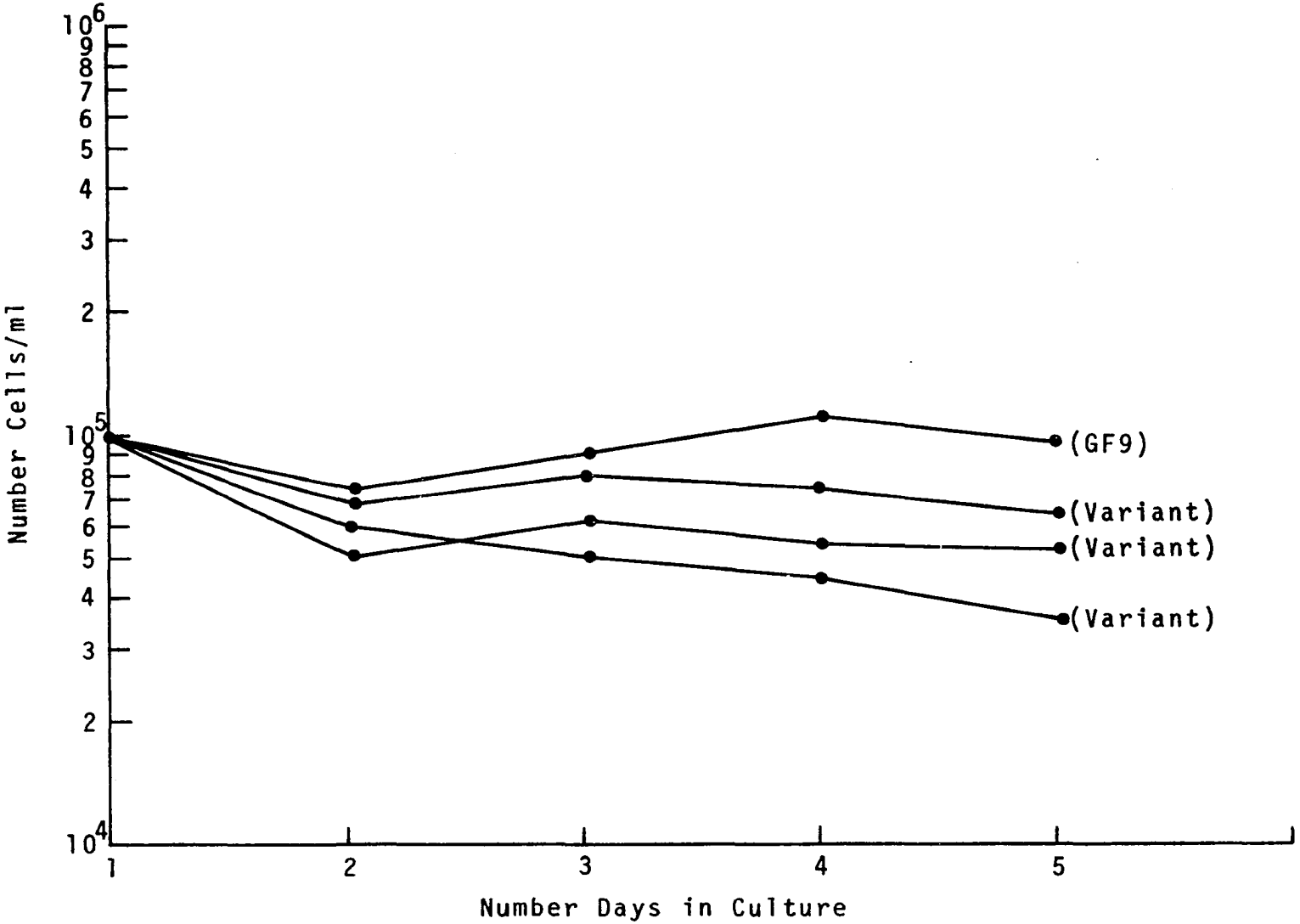


Figure 10. Growth curves of GF9 and "lactose variants" in Fischer's Medium containing 5.5×10^{-6} M glucose and 15 percent dialyzed horse serum


Number Cells/ml
10^6
9
8
7
6
5
4
3
2
10^5
9
8
7
6
5
4
3
2
10^4
1
2
3
4
5
Number Days in Culture
(GF9)
(Variant)
(Variant)
(Variant)

division and maintenance of surrounding cells. This possibility was examined by cloning mutagen-treated cells in Fischer's medium lacking an added carbon source and containing 15 percent dialyzed horse serum at a concentration of 4.0×10^5 cells per tube. If cross-feeding were the causative factor in the appearance of "lactose selection variants", the absence of lactose should not prevent the appearance of variant colonies. Table 8 gives the results of these experiments. Variant colonies were not obtained. These findings suggest that the mutagens did not induce an artificial cell clumping which caused cross-feeding of cells and false appearance of colonies.

The third alternative

The third alternative was that the variant colonies could utilize lactose only in agar culture as opposed to lactose-containing suspension medium. To determine if this was true, 20 clones were isolated, dispersed in 0.5 ml selective medium and immediately recloned in selective medium at a concentration of 10^4 cells per tube. A total of 4.0×10^4 cells were cloned from each variant colony. A GF9 parental clone was isolated, dispersed in 0.5 ml nonselective medium and recloned at 100 cells per tube. Four tubes were cloned.

GF9 yielded an average of 20.25 colonies per 100 cells plated. Although this cloning efficiency was considerably lower than normal (80-90 percent), it indicated that the procedure was practicable. Of the 20 variants recloned, only one gave a positive result. This one variant yielded one colony or an average of 0.25 colonies per 10^4 cells plated. This subclone was reisolated from the cloning medium and dispersed in 0.5 ml

Table 8. Cloning experiments designed to detect cross-feeding in mutagen treated cells

Number Viable Cells Plated	Percent of Plated Cells Surviving in Nonselective Medium[a]	Number of Variant Colonies Recovered in Medium Lacking Carbon source[b]	Frequency of Variant Colonies in Population of Surviving Cells ($\times 10^{-5}$)
6.87×10^7	3.30	0.0	0.0
7.64×10^7	4.20	0.0	0.0
7.00×10^7	3.72	0.0	0.0
8.00×10^7	2.70	0.0	0.0
29.5×10^7	-	-	-
Average values:	3.48	-	-

[a]Fischer's Medium plus 15 percent dialyzed horse serum.

[b]Fischer's Medium minus sugar plus 15 percent dialyzed horse serum.

selective medium. It contained approximately 3.5 x 10^4 viable cells, which were immediately recloned again in selective medium at 10^4 cells per tube. A total of 3.0 x 10^4 cells were plated. No colonies were obtained from this second recloning.

The results of these recloning experiments indicate that the colonies were not variants fully capable of utilizing lactose only in agar culture.

The fourth alternative

The fourth and final alternative considered was that the "lactose selection variants" might have been the result of an unstable "lac^+ condition", i.e., some of the mutagen-treated cells might have been able to utilize lactose for a brief period because of a temporary production of lactase. If it were assumed that the lactase enzyme in mammalian cells is regulated by a repressor substance, such an event could have occurred as the result of: (1) an unstable alteration in the repressor gene; (2) a loss of the chromosome bearing the repressor.

An alteration in the gene that produces the repressor molecule could have inhibited the production of the repressor, or caused the synthesis of a nonfunctional repressor, a repressor incapable of binding to DNA. The occurrence of either of these events would permit the synthesis of lactase.

Chromosomal loss could have caused the temporary production of lactase only if the repressor and structural genes were not on the same chromosome. If this were true, a loss of chromosomes from the cells could mean that the chromosome bearing the repressor gene had been lost. These cells would then be eventually selected against, even in lactose

medium, because of the loss of other vital chromosomal functions.

Neither of the proposed explanations to account for the unstable "lac^+ condition" could be tested experimentally. However, the second proposal was investigated by checking the variants for changes in chromosome number. Ten variants, numbered LA1-LA10, were isolated and their karyotypes analyzed. Analyses were made at one and two weeks after isolation from the selective medium. At that time variants LA1-LA5 had gone through approximately 20 generations, LA6-LA10 about 25-37 generations.

The modal chromosome number for GF9 is 40. Chromosome distribution patterns of GF9 and the variants are given in Figures 11, 12, and 13. Chromosome patterns for LA4 and LA8 are not included; the preparations were lost. Significant differences (determined statistically using the *t* test) were found between the mean chromosome number of the parental cells and variants LA1, LA7, and LA9. Mean chromosome numbers for these variants are 38.8, 38.1, and 36.1 respectively. Also there is an obvious difference between the distribution pattern of GF9 and variants LA3 and LA7. The wider distribution pattern in these variant clones is a clear illustration that there are a larger number of cells with fewer chromosomes. However, these findings alone are not sufficient to conclude that the condition described above actually did occur, but they do suggest that chromosomal instability may have been a means whereby a temporary production of lactase could have occurred.

Assuming that the variants may have been the result of a temporary production of lactase, attempts were made to detect lactase activity in

Figure 12. Histiograms of chromosome per cell against number of cells. For GF9 and each variant 100 cells were counted. The diploid number (2n) of the mouse is 40.

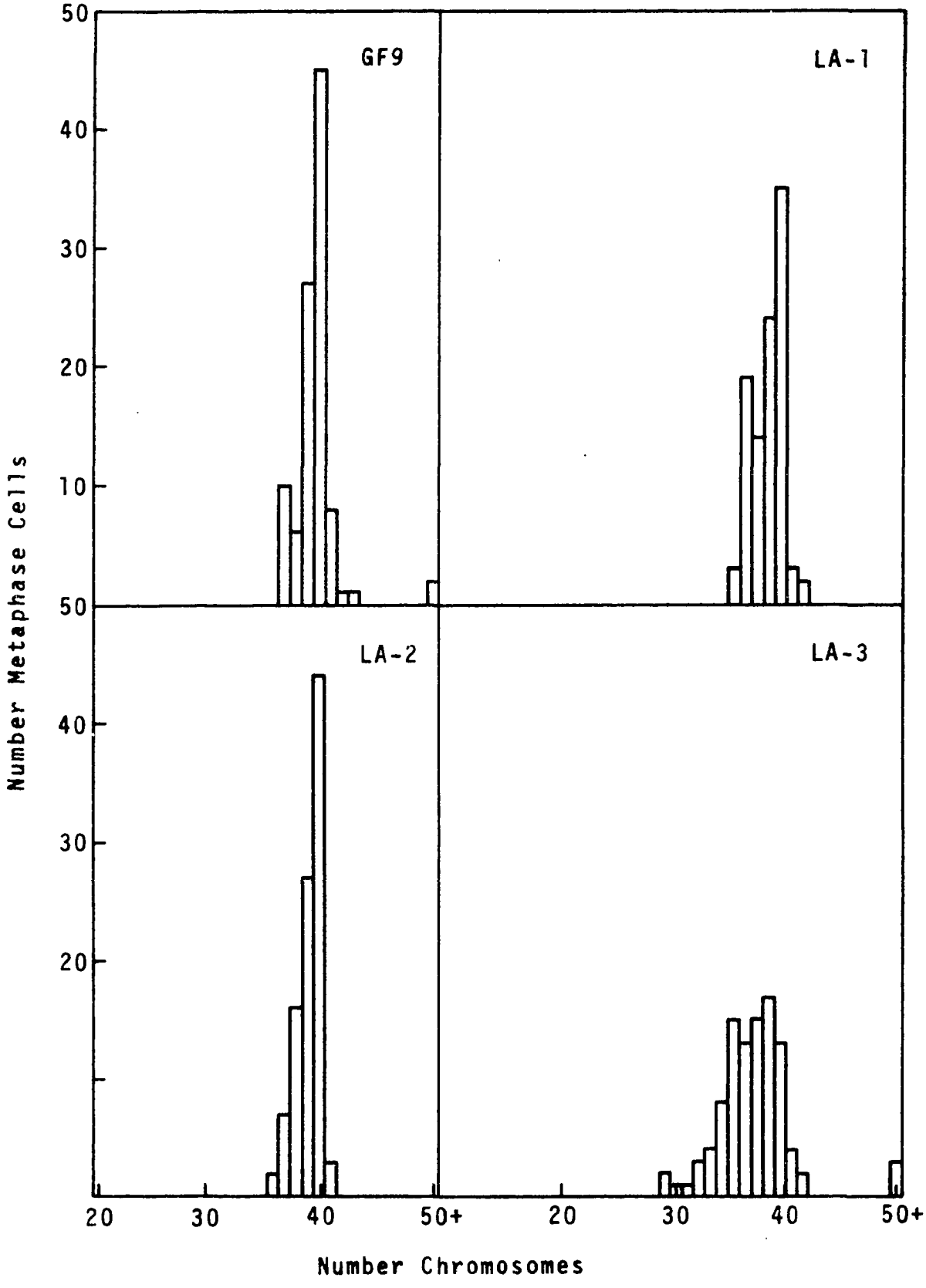

GF9
LA-1
LA-2
LA-3
Number Metaphase Cells
Number Chromosomes
50
40
30
20
10
20
30
40
50+

Figure 11. Histiograms of chromosome per cell against number of cells. For GF9 and each variant 100 cells were counted. The diploid number (2n) of the mouse is 40.

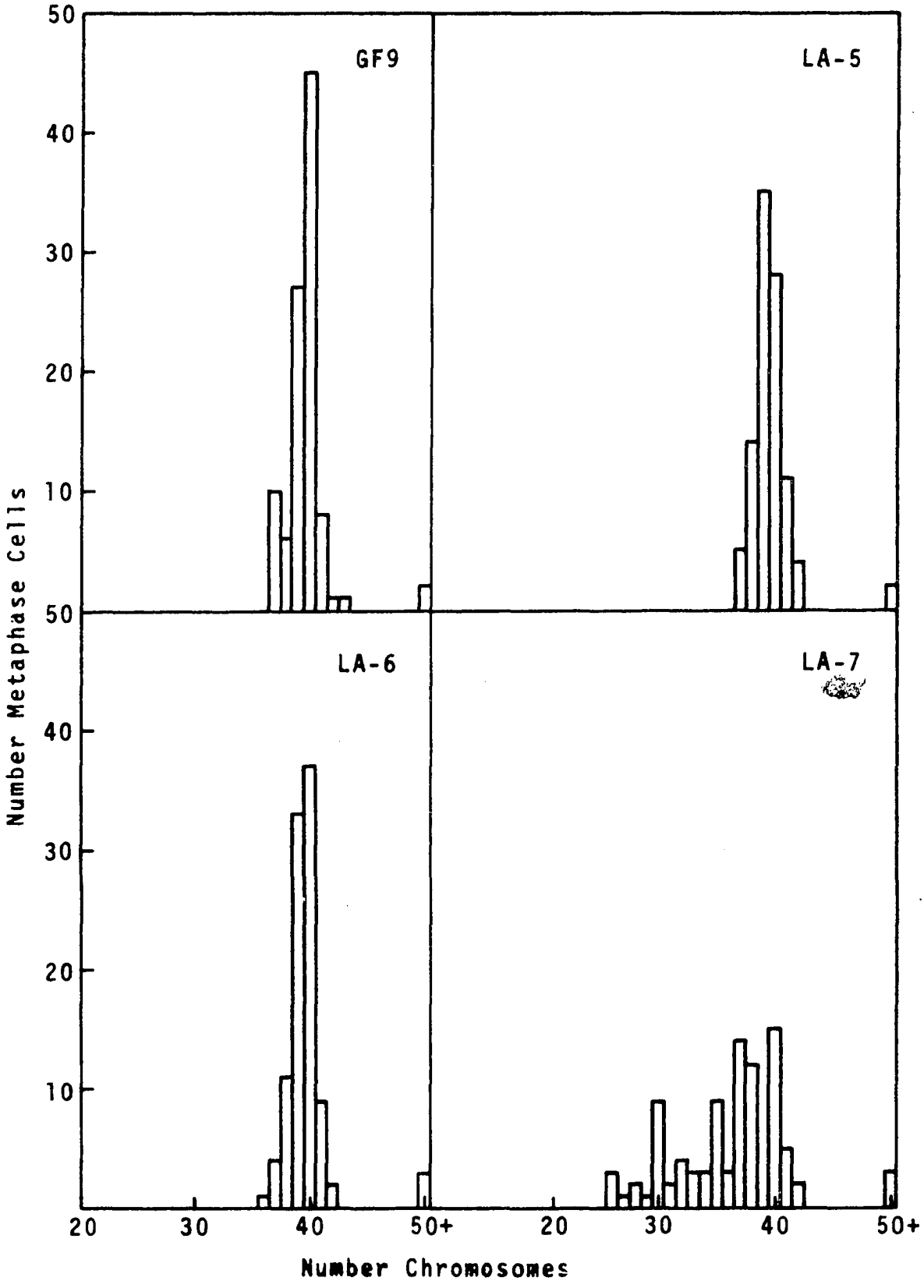
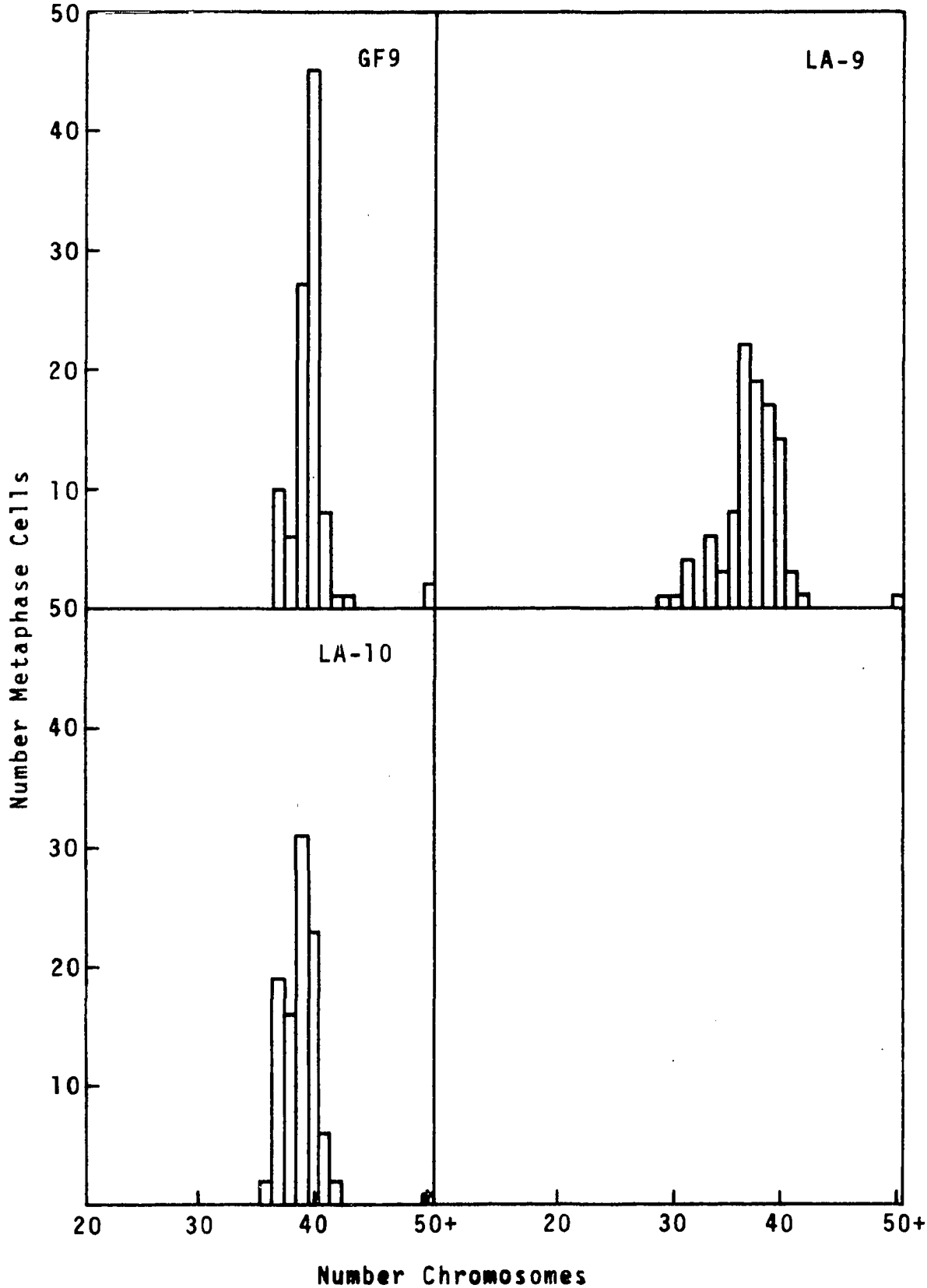

GF9
LA-5
LA-6
LA-7
Number Metaphase Cells
Number Chromosomes
50
40
30
20
10
20
30
40
50+

Figure 13. Histiograms of chromosome per cell against number of cells. For GF9 and each variant 100 cells were counted. The diploid number (2n) of the mouse is 40.


GF9
LA-9
LA-10
Number Metaphase Cells
Number Chromosomes
50
40
30
20
10
20
30
40
50+

single colonies immediately after they became visible in the cloning medium. The variant clones contain approximately 5.0×10^4 cells. These are too few cells to use the lactase assay procedure described in the Materials and Methods section. Therefore, another detection procedure was used.

O-nitrophenyl-B-galactoside (ONPG) is a synthetic beta-galactoside and a substrate of beta-galactosidases in mammals (see Literature Review) and in bacteria. When this compound is hydrolyzed by the enzyme a characteristic yellow color is produced. It was reasoned that if the clones contained lactase and they were placed in a solution of ONPG, the synthetic substrate would be hydrolyzed producing an observable yellow color.

Variant colonies were isolated from the selective cloning medium, washed once with a balanced salt solution, dispersed in 0.5 ml of a 2.0 mM solution of ONPG in a small test tube, and incubated for 1-3 hours at room temperature. Separate incubations were also made at 37° C for the same time period. At the conclusion of the incubation period, the tubes were observed for a color change. Fifty colonies were tested in this manner; a positive reaction was not observed for any of the clones.

Growth Characteristics of Variant Colonies

Growth curves for five of the variants are shown in Figure 14. These curves are typical of the more than 50 colonies tested. The "lactose selection variants" have an average doubling time of 16 to 24 hours which is longer than that of GF9 (10 to 11 hours).

Cloning efficiencies for 10 of the variants are shown in Table 9. These are the variants that were analyzed for a loss of chromosomes.

Figure 14. Growth curves for GF9 and five of the variant colonies in Fischer's Medium plus 15 percent horse serum

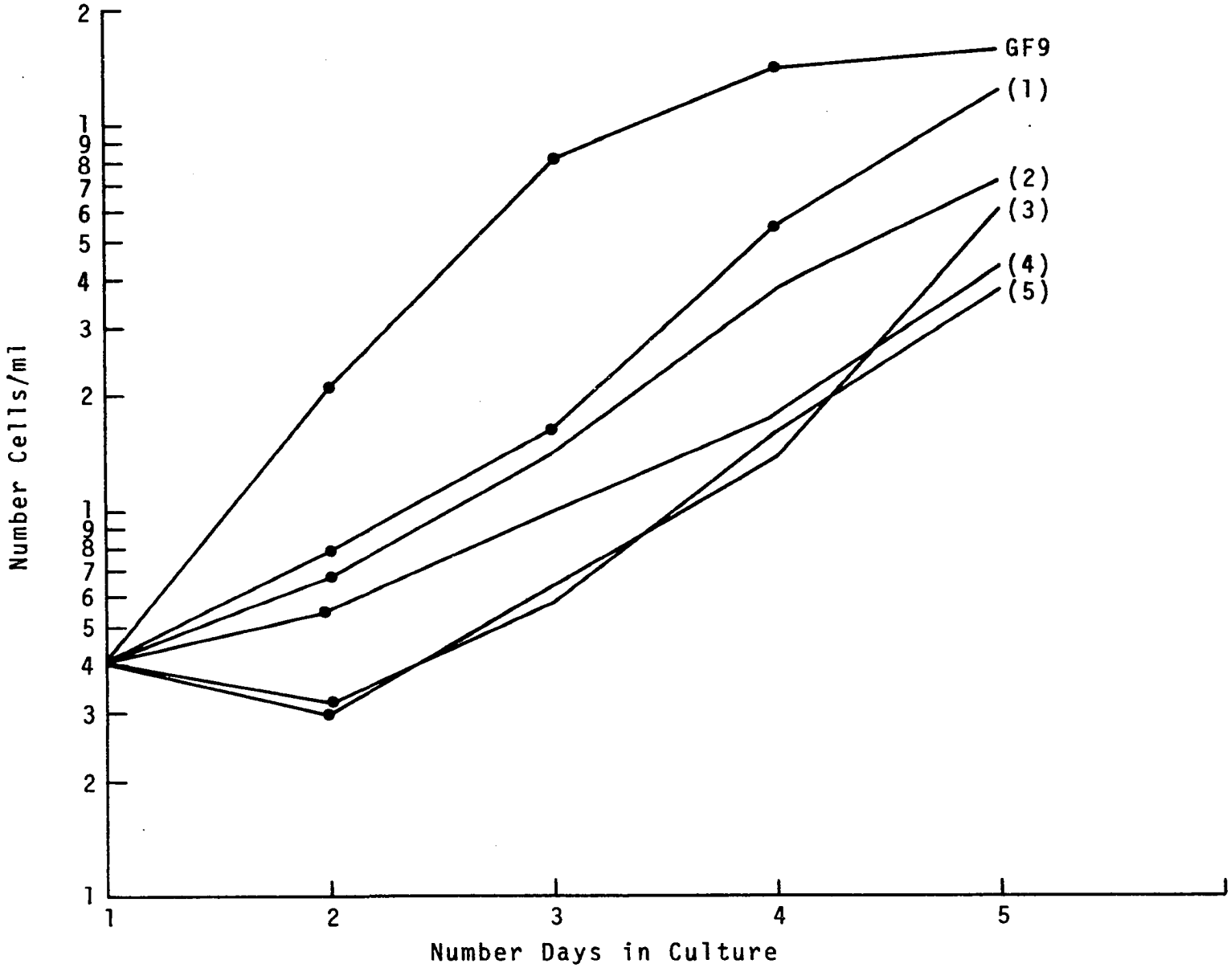

Number Cells/ml
2
1
9
8
7
6
5
4
3
2
1
9
8
7
6
5
4
3
2
1
1
2
3
4
5
Number Days in Culture
GF9
(1)
(2)
(3)
(4)
(5)

Table 9. Cloning efficiencies of "lactose selection variants"[a]

Variant	Percent Cloning Efficiency
LA 1	77.56
LA 2	88.00
LA 3	86.62
LA 4	80.50
LA 5	85.50
LA 6	82.75
LA 7	87.50
LA 8	84.00
LA 9	81.25
LA 10	85.50
GF 9	88.92

[a]Variants were cloned in Fischer's Medium supplemented with 15 percent horse serum at a concentration of 100 cells/tube. Sixteen replicate tubes were plated for each variant. LA 1-LA 5 were cloned at approximately 20 generations; LA 6-LA 10 at approximately 25-37.

Cloning efficiencies for these clones ranged from 77.56-87.50 percent. The parental cells have a cloning efficiency of 80-90 percent.

Effect of BUDR and DMSO on GF9 Cells

The dose response curves for BUDR and DMSO are shown in Figures 15 and 16 respectively. Cells grown in medium containing 3 ug/ml BUDR and three percent DMSO multiplied at approximately the same rate as those of the control cultures. In cultures treated with these agents no morphological changes were observed. In separate experiments, BUDR (3 ug/ml) and DMSO (three percent) were added to selective medium containing different concentrations of lactose in an attempt to induce the synthesis of lactase. The results of these tests are given in Figures 17 and 18. Viable cell counts were made daily. No cell growth was observed; this indicated the absence of lactase activity. The cultures were maintained until no viable cells were noted. This eliminated the possibility that only a small subpopulation of cells may have been capable of growing in medium containing BUDR and lactose and in medium containing DMSO and lactose.

Induction Experiments With Intestine 407, GF9 and Variants

Embryonic Intestine 407 is a small intestinal epithelial cell line and therefore must certainly retain the genetic potential necessary for the production of lactase. However, this cell line will not grow in medium containing lactose and dialyzed horse serum, indicating the absence of any functional lactase activity. Since this cell line is naturally expected to have lactase activity, it appears that the enzyme has been

Figure 15. Dose response curves of GF9 cells in Fischer's Medium containing different concentrations of BUDR and 15 percent horse serum

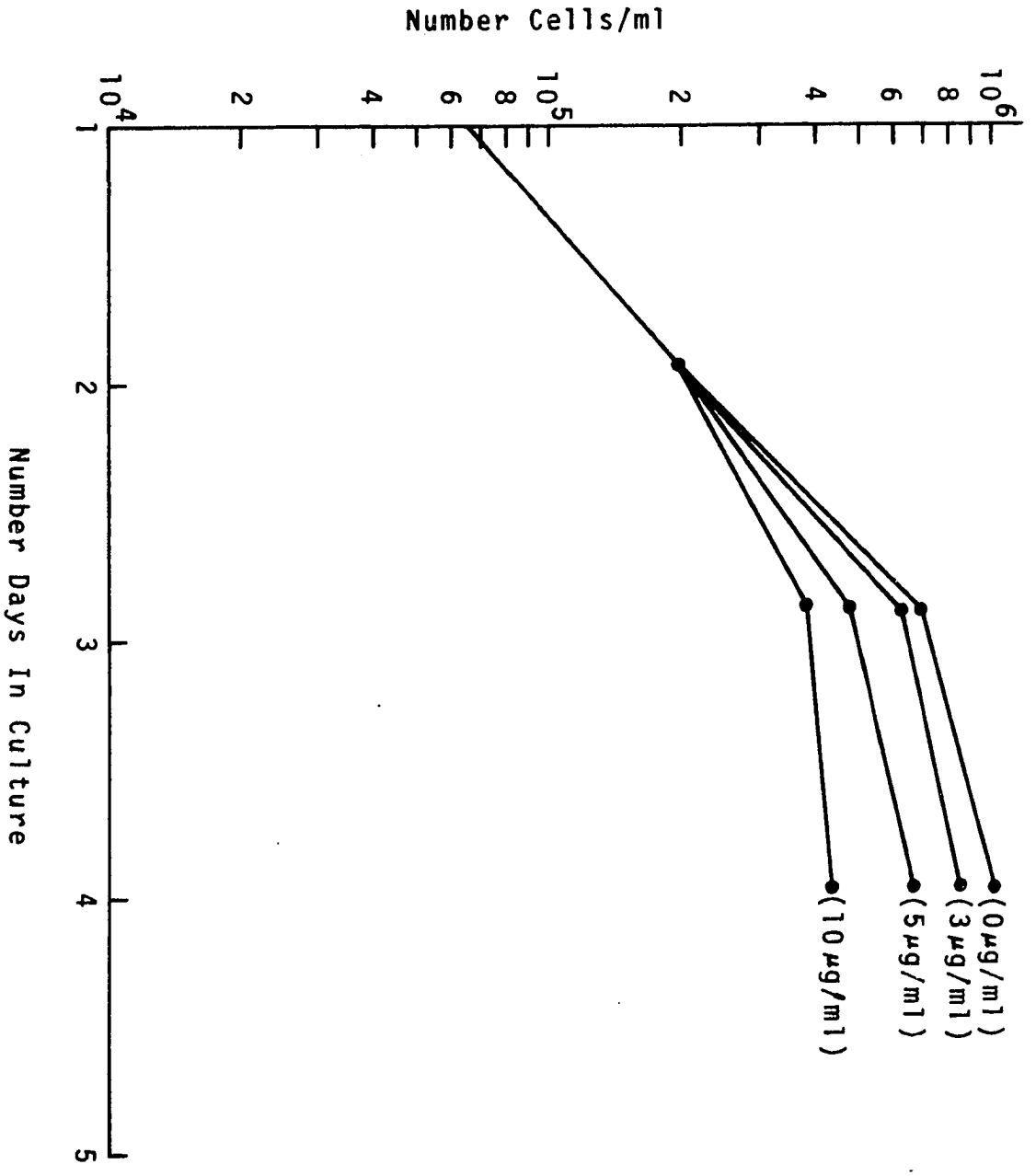

Number Cells/ml
10^4
2
4
6
8
10^5
2
4
6
8
10^6
1
2
3
4
5
Number Days In Culture
(0 µg/ml)
(3 µg/ml)
(5 µg/ml)
(10 µg/ml)

Figure 16. Dose response curves of GF9 cells in Fischer's Medium containing different concentrations of DMSO and 15 percent horse serum

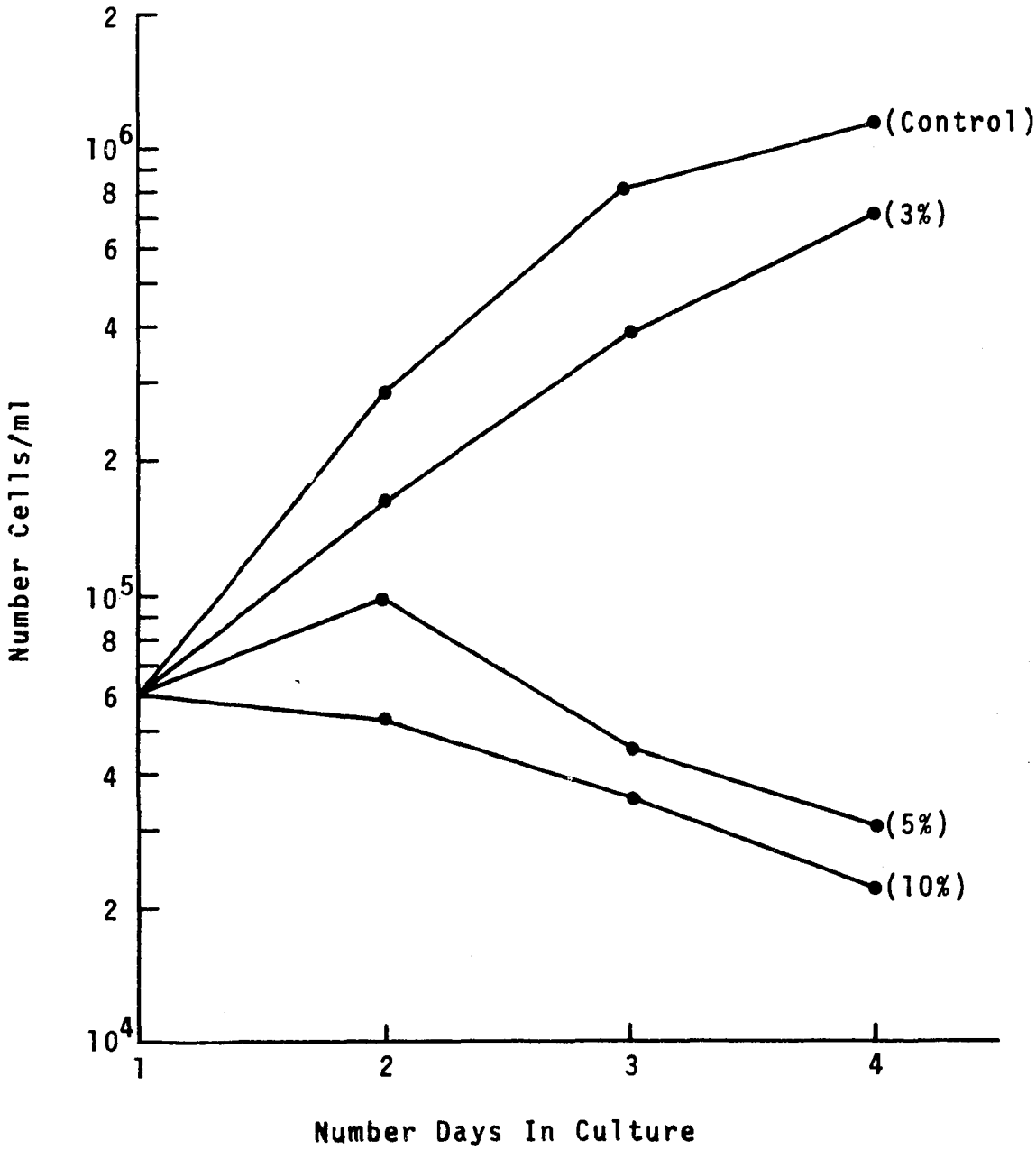

2
10^6
8
6
4
2
10^5
8
6
4
2
10^4
1
2
3
4
(Control)
(3%)
(5%)
(10%)
Number Cells/ml
Number Days In Culture

Figure 17. Growth curves of GF9 cells in Fischer's Medium containing glucose, 3 ug/ml BUDR and 15 percent dialyzed horse serum; and in Fischer's Medium containing lactose, 3 ug/ml BUDR and 15 percent dialyzed horse serum

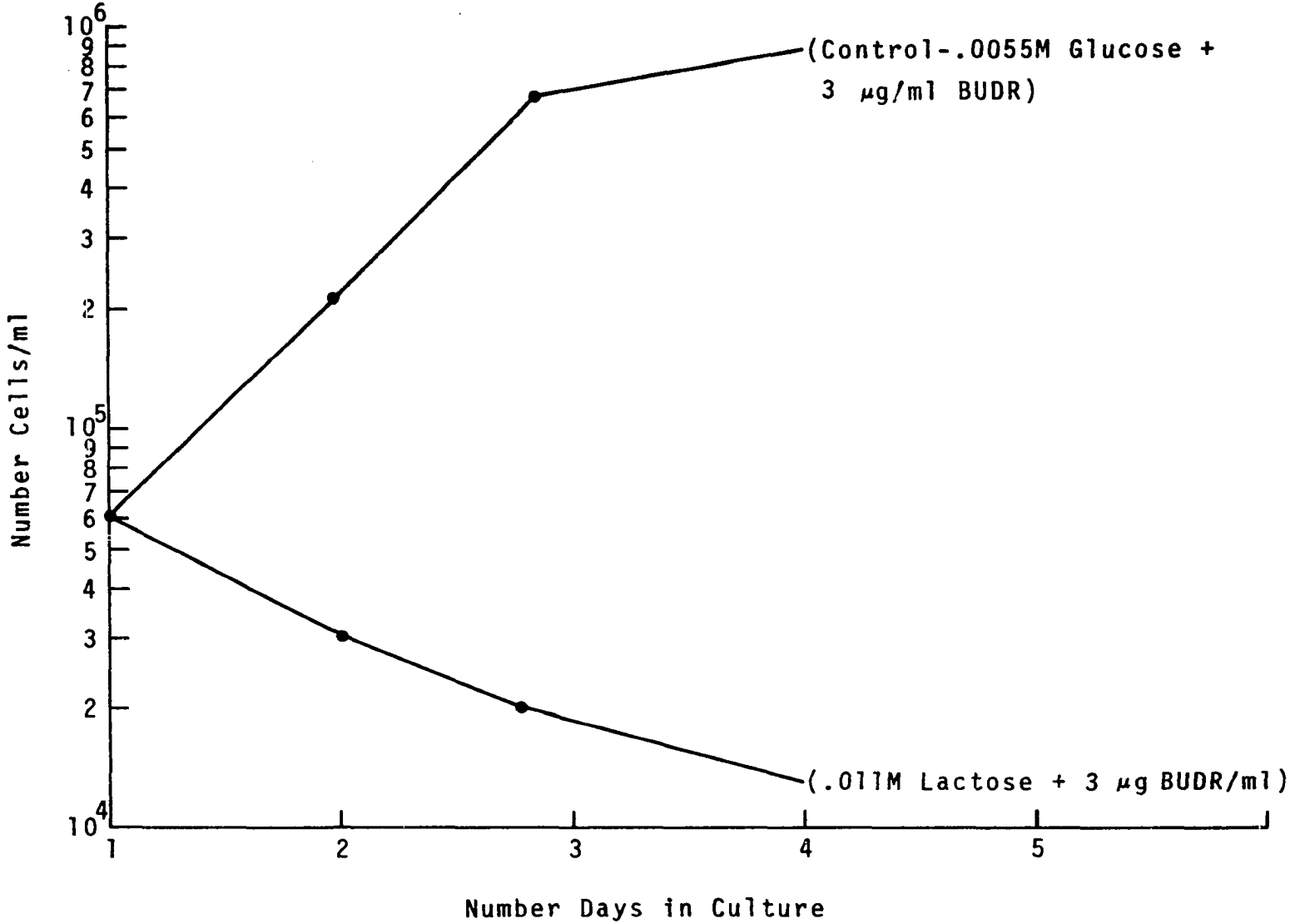
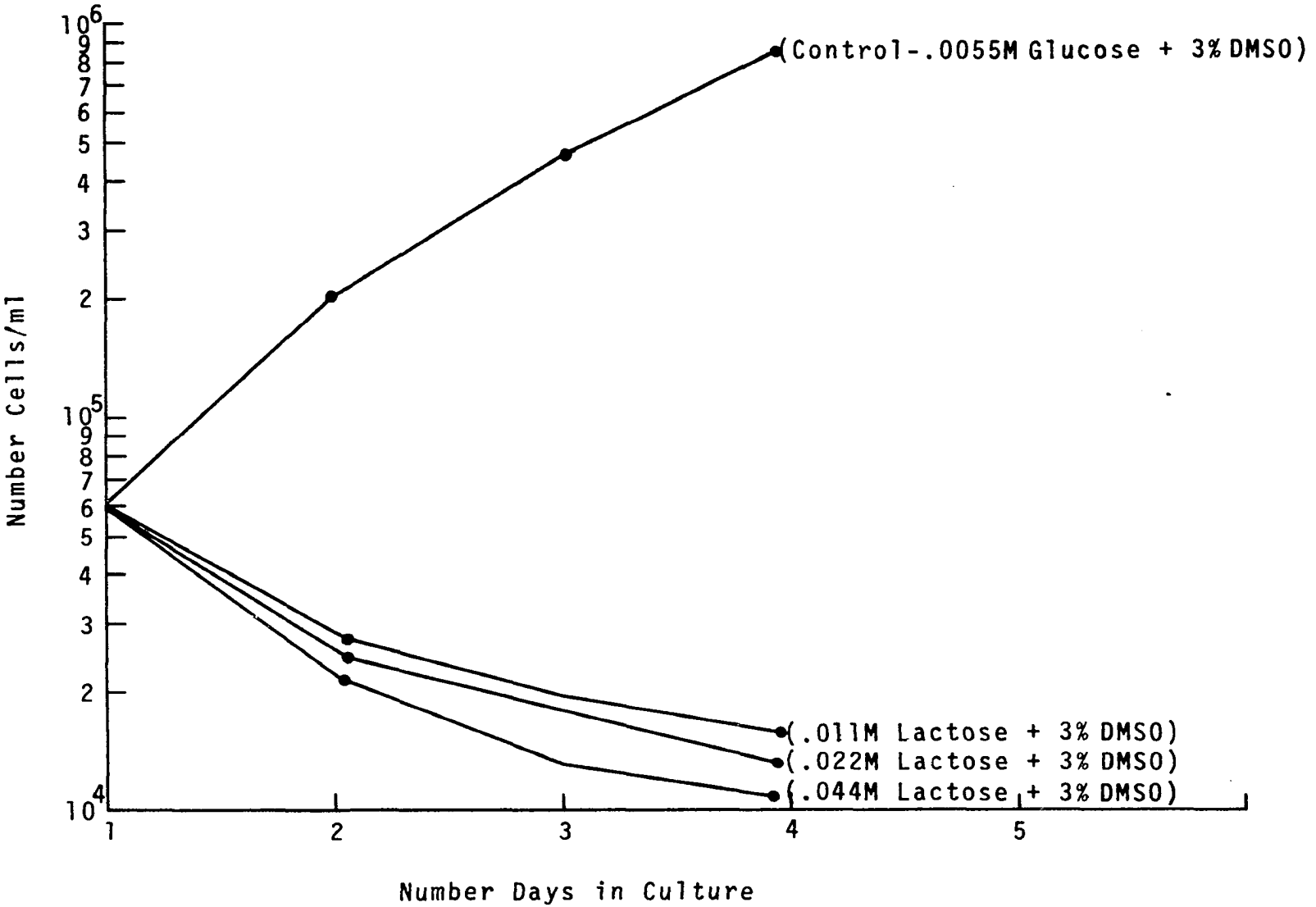

Number Cells/ml
10^6
9
8
7
6
5
4
3
2
10^5
9
8
7
6
5
4
3
2
10^4
1
2
3
4
5
Number Days in Culture
(Control-.0055M Glucose + 3 μg/ml BUDR)
(.011M Lactose + 3 μg BUDR/ml)

Figure 18. Growth curves of GF9 cells in Fischer's Medium containing glucose, 3 percent DMSO, and 15 percent dialyzed horse serum; and in Fischer's Medium containing different concentrations of lactose, 3 percent DMSO and 15 percent dialyzed horse serum


Number Cells/ml
10^6
9
8
7
6
5
4
3
2
10^5
9
8
7
6
5
4
3
2
10^4
1
2
3
4
5
Number Days in Culture
(Control-.0055M Glucose + 3% DMSO)
(.011M Lactose + 3% DMSO)
(.022M Lactose + 3% DMSO)
(.044M Lactose + 3% DMSO)

"turned off" or perhaps it was never "turned on" (this cell line was established from a two month old embryo; lactase is not present in the human fetus until around the middle of gestation).

Attempts were made to induce the lactase enzyme in Intestine 407 using lactose and isopropyl thiogalactoside. These agents are known inducers of the beta-galactosidase enzyme in Escherichia coli (Jacob and Monod, 1961). The results are given in Table 10. Lactase was not induced in 407 with these compounds.

The same agents were used in induction experiments with GF9 and some of the "lactose selection variants" (Table 10). The results again were negative.

A Fetal Mouse Small Intestinal Cell Line

All attempts to establish a line of small intestinal epithelial cells from fetal mice were futile. The cultures consisted of two types of cells; fibroblast and epithelial cells. After approximately 2-3 days in culture, epithelial cells were no longer present. Fibroblasts were maintained in culture for several weeks but never grew to give a confluent monolayer of cells. Attempts were made to select for epithelial cells initially by placing the cells directly in medium containing lactose and dialyzed horse serum. In this medium the cells did not attach to the surface of the culture flask and did not grow. Thus, the cells were placed directly in medium containing glucose and regular horse serum and allowed to attach to the culture flask. Three to four days later this medium was removed and medium containing lactose and dialyzed horse serum was added. The

Table 10. Induction experiments

Cells Used	Inducer	Concentration of Inducer	Length of Induction	Lactase Activity[a]
Intestine 407	None	-	-	0.0
	Lactose	.011M	12 hrs.	0.0
		.022M	12 hrs.	0.0
	IPTG	10^{-3}M	12 hrs.	0.0
		10^{-2}M	12 hrs.	0.0
GF 9	None	-	-	0.0
	Lactose	.011M	6 hrs.	0.0
		.022M	6 hrs.	0.0
	IPTG	10^{-3}M	6 hrs.	0.0
		10^{-2}M	6 hrs.	0.0
Variant Colonies[b]	None	-	-	0.0
	Lactose	.011M	6 hrs.	0.0
		.022M	6 hrs.	0.0
	IPTG	10^{-3}M	6 hrs.	0.0
		10^{-2}M	6 hrs.	0.0
Control for Assay:				
Extract from Mouse Intestine	-	-	-	3.8

[a]Expressed as Moles disaccharide hydrolyzed/min.

[b]A total of 20 variant colonies were tested separately.

cells did not grow in this medium and became detached from the surface of the flask. This indicated that these cells were not capable of utilizing lactose and that there was no lactase present. It appeared that these cells are capable of undergoing only a finite number of divisions in vitro. Assuming this to be true, attempts were made to increase the proliferation of these cells by transforming them with SV-40 virus. This also proved to be unsuccessful. Increased numbers of fibroblasts were obtained but epithelial cells were still only present for 1-3 days in culture. These cultures were unable to utilize lactose. Table 11 outlines the procedures used in the attempt to establish a line of epithelial cells from fetal mouse intestine in culture and gives the results of each variation in technique.

Table 11. Outline of procedures used to establish a small intestinal epithelial cell line from fetal mice

Procedures	Results
I. Isolation of small intestinal tissue from fetal mice and dispersion of the tissue with trypsin	A suspension of single cells.
II. Establishment of cultures	
A. Cells plated directly in selective medium.[a]	Cells did not attach to the surface of the culture flask.
B. Cells plated directly in nonselective medium.[b]	Cells became attached to the culture flask surface. Cultures consisted of two cell types, morphologically similar to fibroblasts and epithelial cells.
These cultures were treated in two ways:	
1. Medium removed. Cells washed once with a balanced salt solution and selective medium added.	Cells became detached from the surface of the culture flask.
2. Cells remained on nonselective medium.	A confluent monolayer of cells was not obtained.

[a]Selective medium. Eagle's basal medium with Hank's salts containing 0.011 lactose and 15 percent dialyzed horse serum.

[b]Nonselective medium. Eagle's basal medium with Hank's salts containing 0.0055M glucose and 15 percent dialyzed horse serum.

Table 11 continued

Procedures	Results
	Epithelial-like cells were present for only 1-3 days in culture. Fibroblasts-like cells were present for approximately two weeks.
C. Cells inoculated with 0.5 and 0.25 ml SV-40 virus in nonselective medium[c]	Cells became attached to surface of culture flask.
These cultures were treated in two ways:	
1. Medium removed. Cells washed once with a balanced salt solution and selective medium added.	Cells became detached from the surface of the culture flask.
2. Medium removed. Cells washed once with a balanced salt solution and nonselective medium added.	Confluent monolayer of cells obtained. Number fibroblast-like cells increased greatly. No increase in the number of epithelial-like cells was observed.

[c]Titer of virus = $10^{6.3}$ $TCID_{50}$/.1 ml.

DISCUSSION

The objective of this study was to establish a mammalian cell line, in culture, that actively produced the lactase enzyme. It was hoped that such a cell system would provide a means whereby the biochemical properties and the genetic regulation of this intestinal disaccharidase could be thoroughly investigated. Three approaches were used to accomplish this objective:

1. Attempts were made to induce lactase activity in Intestine 407, a previously established small intestinal epithelial cell line, and in GF9, an established white blood cell line, by using lactose and isopropyl-thiogalactoside.
2. Attempts were made to establish a small intestinal epithelial cell line from fetal mice in culture.
3. Attempts were made to reverse natural lactase differentiation in GF9 cells by exposing these cells to mutagens and agents known to affect differentiation in other cell types.

Lactase activity was not induced in Intestine 407 and GF9 cells using lactose and isopropyl-thiogalactoside, known inducers of beta-galactosidase in bacterial systems (Jacob and Monod, 1961). Although human and animal in vivo studies suggest that lactase is not inducible by lactose, this conclusion cannot be drawn from my experiments because it is possible that the system for transport of lactose into GF9 cells is defective.

When the beta-galactosidase gene is activated in Escherichia coli,

another gene is also induced. This gene codes for the production of a molecule termed the "M protein" (Jacob and Monod, 1961). A small number of "M protein" molecules are always present in the cell (basal level), but the high induced levels of this protein facilitate the uptake of lactose by the bacterial cell. In the absence of this protein the bacterial cell cannot incorporate lactose. It is possible that a similar protein is required for lactose uptake in mammalian cells, and that the gene coding for this protein was not activated by the methods employed.

Furthermore, the procedure used may have been too simple. Perhaps there are other factors that play a role in the activation of the enzyme in mammalian cells. In E. coli the transcription of the beta-galactosidase gene requires cyclic AMP (3', 5'-cyclic adenylic acid) (Zubay and Beckwith, 1970).

Lactase activity in the mouse is maximal immediately after birth and decreases dramatically after weaning (2-3 weeks later). It was expected that epithelial intestinal cells from fetal mice would retain their ability to synthesize lactase in vitro. However, all attempts to establish a permanent line of these cells in culture were unsuccessful (Table 11). The selective medium containing lactose did not support growth of any cell type isolated.

Other workers (Harrer et al., 1964; Stern and Jensen, 1966; Browning and Trier, 1969) have also been unable to establish small intestinal epithelial cells in culture. The cells of the small intestine are known to have a very short life span even in vivo. This may account for failures in attempts to establish these cells in culture, i.e., it is possible that

the epithelial cells have been genetically programmed to undergo only a finite number of divisions. There are cell types for which this is true. For example, fibroblasts are capable of undergoing only 37 passages (111 generations) in vitro. These cells can become permanent lines only if they are transformed; transformation is presumably always the result of viral infection. Attempts were made in this study to purposely transform the epithelial cells with SV-40 virus. There was no evidence of transformation of these cells following infection with this virus. In future studies it may be worthwhile to try transforming these intestinal cells with other viruses; for example, Rous Sarcoma and Polyoma. SV-40 virus has been used successfully in the purposeful transformation of fibroblast cells (Todaro and Green, 1964).

Treatment of GF9 cells with the base substitution mutagens, EMS and NG, yielded a total of 3707 colonies in selective medium, i.e., medium in which lactose was the only carbon source provided. Fourteen hundred of these putative mutants were isolated and subcultured immediately in liquid selective medium to test their ability to utilize lactose in suspension culture. None of the tested colonies proved to be true lac^+ mutants, i.e., cells capable of using lactose as a carbon source. Although only 1400 of the 3707 recovered colonies were screened in this manner, it is not probable that the remaining 2307 colonies contained any true lac^+ mutants. Assuming that there might have been such a mutant in this population of clones, it would have to occur at a very low frequency.

The colonies which appeared after treatment with mutagens were referred to as "lactose selection variants". Control experiments (Table 6)

showed that these variants did not arise spontaneously, but were the result of mutagenic action.

The selection system used in this part of the study was very straightforward and should have given rise to clones of the desired phenotype, i.e., the permanent acquisition of the ability to utilize lactose. Since this was not the case, attempts were made to determine the event(s) responsible for the appearance of "lactose selection variants" in the selective medium. Four possibilities were considered:

1. The variant colonies may have resulted from growth on residual glucose or galactose in the selective medium.
2. The variants may have appeared as a result of cross-feeding between cells cloned as clumps rather than as individual cells.
3. The variant colonies may have been capable of utilizing lactose only in agar culture and therefore were unable to grow in suspension culture.
4. The variants may have appeared because of an unstable "lac condition" in which some of the mutagenized cells were able to utilize lactose for a brief period due to a temporary production of lactase.

Each of these suggested events for the appearance of "lactose selection variants" was examined. The variants did not result from growth on residual glucose or galactose in the selective medium since neither of these monosaccharides could be detected by any of the methods employed. Mutagen treated cells were cloned in a medium lacking an added carbon source (Table 8); no colonies were obtained. Therefore, the variants were

not the result of cross-feeding. Also the "lactose selection variants" were not capable of utilizing lactose only in agar culture. This was evident when these variants could not be recloned in the selective cloning medium immediately following isolation. However, it seems that the variants may have appeared in the selection system because of a chromosomal imbalance (Figures 10-12) which, in theory, may have allowed a transitory utilization of the carbon source (probably only lactose) provided.

In regard to the latter conclusion, it was shown that there were significant differences in both chromosome number and patterns of distribution between the parental cells and four of the eight variants tested. This was the only abnormal event correlating with the appearance of "lactose selection variants" which could be detected.

If mutagen-induced chromosomal instability was the event responsible for the appearance of the "lactose selection variants", and it is assumed that lactose was the only carbon source available (this is likely from data presented), one might hypothesize that this chromosome instability might have caused a temporary ability to utilize lactose.

Attempts were made to detect the enzyme in single clones as soon as they became visible in the selective cloning medium using ONPG as a substrate; no enzyme activity was observed. However, this observation may not be significant since the studies of Gray and Santiago (1969) show that mammalian lactase has very little capability of hydrolyzing synthetic substrates.

Terzi (1974) encountered a similar situation of chromosomal imbalance in attempting to isolate bromodeoxyuridine and thioguanine

resistant mutants from a line of Syrian Hamster cells. This investigator found that the putative mutants isolated from his selection system were not true drug resistant mutants, but were mutagen-induced variants that had been able to grow in the selective medium because of a variation in chromosome number. These pseudo drug resistant mutants were characterized by a high phenotypic reversion rate (drug resistance → drug sensitivity).

A high phenotypic reversion rate ($lac^+ \rightarrow lac^-$) would explain why the variants isolated in this study were not able to utilize lactose in suspension culture following their isolation from the selective cloning medium. This high reversion rate is probable if it is assumed that the lac^+ phenotype was due to abnormal chromosome numbers and that among the mutagen treated cells there is a natural selection for cells with normal karyotypes. Cells with low chromosome numbers would be eliminated quickly in lymphocyte cultures. If so, this would also explain why only 50 percent and not 100 percent of the variants analyzed had chromosomal abnormality.

This study is only the second in which a chromosomal loss or instability has been found to give rise to a selected phenotype following mutagenesis. This finding is an important one in that it helps define the necessary parameters in the process of obtaining true mutants in mammalian cells.

Bromodeoxyuridine and dimethyl sulphoxide, two chemicals which have been shown to inhibit and induce differentiation (see Literature Review) in a number of <u>in vitro</u> cell systems, had no effect on GF9 cells. It

appears that these cells are not susceptible to the actions of these agents.

Therefore, in conclusion, using the three approaches outlined above, I was not able to establish a mammalian cell line in culture that actively produces the lactase enzyme. This study, to my knowledge, represents the first attempt to provide an in vitro system for the study of the mechanisms controlling the lactase enzyme in mammalian cells.

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